Regulation of non-antioxidative and inflammatory pathways in macrophages by long-chain metabolites of α-tocopherol

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3T3-L1	3-Day transfer, inoculum 3 x 10^5 cells, adipocyte-like cells
A549	Adenocarcinomic human alveolar basal epithelial cells
ABC	Adenosine triphosphate (ATP) binding cassette
ACAT	Acetyl-Coenzym A-Acetyltransferase
ADP	Adenosine diphosphate
ADRP	Adipophilin, synonym: Perilipin 2 (PLIN2)
Akt	Protein Kinase B; Ak: temporary classification name for a mouse bred; t: thymoma
AP	Activator protein
ApoE	Apolipoprotein E
ATBC	Alpha-Tocopherol, Beta-Carotene Cancer Prevention
ATF	Activating transcription factor
AVED	Ataxia with Vitamin E Deficiency
B16	Murine melanoma cells
BEAS-2B	Human bronchial epithelial virus transformed cell line
C57BL/6J	Transgenic mice strain
CAR	Constitutive active/androstane receptor
CD	Cluster of differentiation
CDMDHC	Carboxydimethyldecylhydroxychromanol
CDMOHC	Carboxydimethyloctylhydroxychromanol
CE	Cholesteryl ester
CEH	Cholesteryl ester hydrolase
CEHC	Carboxyethylhydroxychromanol
CETP	Cholesteryl ester transfer protein
CHAOS	Cambridge Heart Antioxidant Study
CMBHC	Carboxymethylbutylhydroxychromanol
CMHHC	Carboxymethylhexylhydroxychromanol
COX	Cyclooxygenase
CRAL_TRIO	CRAL: cellular retinaldehyde, TRIO: triple functional domain protein

CVD	Cardiovascular disease
CYP	Cytochrome P450
Db/Db	Leptin receptor deficient knockout mice
DGE	German Society of Nutrition, German: *Deutsche Gesellschaft für Ernährung*
DMSO	Dimethyl sulfoxide
EC	Endothelial cells
EC_{50}	Half maximal effective concentration
ELISA	Enzyme-linked immunosorbent assay
EOC-2O	Brain microglia *Mus musculus* cells
ERK	Extracellular-signal regulated kinases
FABPpm	Fatty acid binding protein in the plasma membrane
FAT	Fatty acid translocase
FATP	Fatty acid transporter protein
FC	Free cholesterol
Fos	Finkel-Biskis-Jinkins (FBJ) osteosarcoma
GC/MS	Gas chromatography mass spectrometry
GPCR	G-protein-coupled receptors
GSH	Glutathione
GSK	Glycogen synthase kinase
GST	Glutathione S-transferases
GW9662	Peroxisome proliferator-activated receptor (PPAR) γ antagonist
$G\alpha_{i2}^{-/-}$	Heterotrimeric G protein knockout mice
HATS	High density lipoprotein (HDL) Atherosclerosis Treatment Study
HCA-SMC	Primary human coronary artery smooth muscle cells
HDAC	Histone deacetylase
HDL	High density lipoprotein
HepG2	Human hepatocellular carcinoma cell line
HETE	Hydroxyeicosatetraenoic acid
HGA	Homogentisate
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A

HOPE-TOO	Heart Outcomes Prevention Evaluation - The Ongoing Outcomes
HPLC	High-performance liquid chromatography
hTAP	Recombinant human tocopherol associated protein
ICM	Intermediate-chain metabolite
IL	Interleukin
INF	Interferon
iNOS	Inducible nitric oxide synthase, synonym: nitric oxide synthase 2
IRF	Interferon regulatory factor
IU	International unit
JAK	Janus kinase
Jun	Gene of avian sarcoma virus 17 (Japanese: ju-nana)
LC/MS-QTOF	Liquid chromatography mass spectrometry quadrupole time-of-flight
LCM	Long-chain metabolite
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LEF	Lymphoid enhancer-binding factor
LOX	Lipoxygenase
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
LRP	Low density lipoprotein receptor-related protein
LTB_4	Leukotriene B_4
LXR	Liver X receptor
Lyn	Lck/Yes novel tyrosine kinase
M1	Classical activated macrophages
M2	Alternative activated macrophages
MAPK	Mitogen-activated protein kinases
MDR	Multidrug resistance
MDT	Marine derived tocopherols
mmLDL	Minimally oxidized LDL
MPO	Myeloperoxidase
MRC/BHF	Medical Research Council/British Heart Foundation

mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance-related proteins
MyD	Myeloid differentiation primary response gene
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NPC1L1	Niemann-Pick C1-like protein 1
oxLDL	Oxidized low density lipoprotein
PAF	Platelet-activating factor
PARP	Poly(adenosin-diphosphate-ribose)polymerase
PC-3	Prostate cancer cells
PC-8	Plastochromanol-8
PG	Prostaglandin
Pgp	P glycoprotein
PK	Protein kinase
PL	Phospholipase
PLTP	Phospholipid transfer protein
PP	Protein phosphatase
PPAR	Peroxisome proliferator-activated receptor
PUFA	Poly unsaturated fatty acids
PXR	Pregnane X receptor
RAEC	Rat aortic endothelial cells
Raf	Rapidly accelerated fibrosarcoma
RANTES	Synonym for Chemokine (C-C motif) ligand 5
Rap	Rat sarcoma (Ras)-related protein
Ras	Rat sarcoma
RAW264.7	Murine leukemia virus transformed macrophages
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reaction

RXR	Retinoid X receptor
SCM	Short-chain metabolite
SELECT	Selenium and Vitamin E Cancer Prevention Trial
SMC	Smooth muscle cells
SPACE	Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease
SPF	Supernatant protein factor
SR	Scavenger receptor
SREBP	Sterol regulatory element-binding protein
STAT	Signal transducers and activators of transcription
SULT	Sulfotransferases
SV-40	Simian vacuolating virus 40
SW480	Human colon cancer cell line
T3	Tocotrienol
TAP	Tocopherol associated protein
TBP	Tocopherol-binding protein
TCF	T cell factors
TE	Tocopherol equivalents
THP-1	Human leukemia monocyte cell line
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOH	Tocopherol
TRIF	Toll-interleukin 1 receptor (TIR)-domain-containing adapter-inducing interferon-β, synonym: TIR-domain-containing adapter molecule (TICAM)
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Tx	Thromboxane
UGT	Uridine diphosphate (UDP)-Glucuronosyltransferase
US	United states
VLDL	Very low density lipoprotein
WACS	Woman's Antioxidant Cardiovascular Study
WHS	Woman's Health Study

Wnt	W: Wingless, nt: integration 1
α-13'-COOH	13'-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyltridecanoic acid; in brief: carboxychromanol
α-13'-OH	13'-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyltridecanol, in brief: hydroxylchromanol
α-TA	α-Tocopheryl acetate
α-TP	α-Tocopheryl phosphate
α-TS	α-Tocopheryl succinate
α-TTP	α-Tocopherol transfer protein

1 Introduction

1.1 Vitamin E

1.1.1 Vitamin E isomers and related structures

Evans and Bishop first described in 1922 the relevance of vitamin E in reproduction of rats (EVANS & BISHOP 1922), which characterized this group of tocopherols (TOH) and tocotrienols (T3) including their α-, β-, γ- and δ-isomers as a vitamin. Nowadays the German Society of Nutrition (Deutsche Gesellschaft für Ernährung, DGE) recommends a daily intake of 12-15 mg/d vitamin E for adults, which should be higher during pregnancy (13 mg) and breast feeding (17 mg). Generally the recommended intake of vitamin E correlates with the amount of poly unsaturated fatty acids (PUFAs) in food: 1 g of diene fatty acid or rather diene equivalent requires a 0.5 mg *RRR*-α-TOH intake.

Vitamin E belongs to the group of fat-soluble vitamins and occurs dominantly in oily plants; therefore nuts, seeds and oils are vitamin E rich sources. Almonds, hazelnuts, germ oil, sunflower oil mainly contains high amounts of α-TOH while walnut, palm oil and soybean predominantly contains γ-TOH (CARDENAS & GHOSH 2013). Tocotrienols are widely distributed in some cereals, in palm oil and rice bran oil. Additionally coconut oil, cocoa butter, soybeans, barley and wheat germ are relevant natural sources for T3s (WONG & RADHAKRISHNAN 2012). Whereas vegetables and fruits – except dried apricots, some legumes,

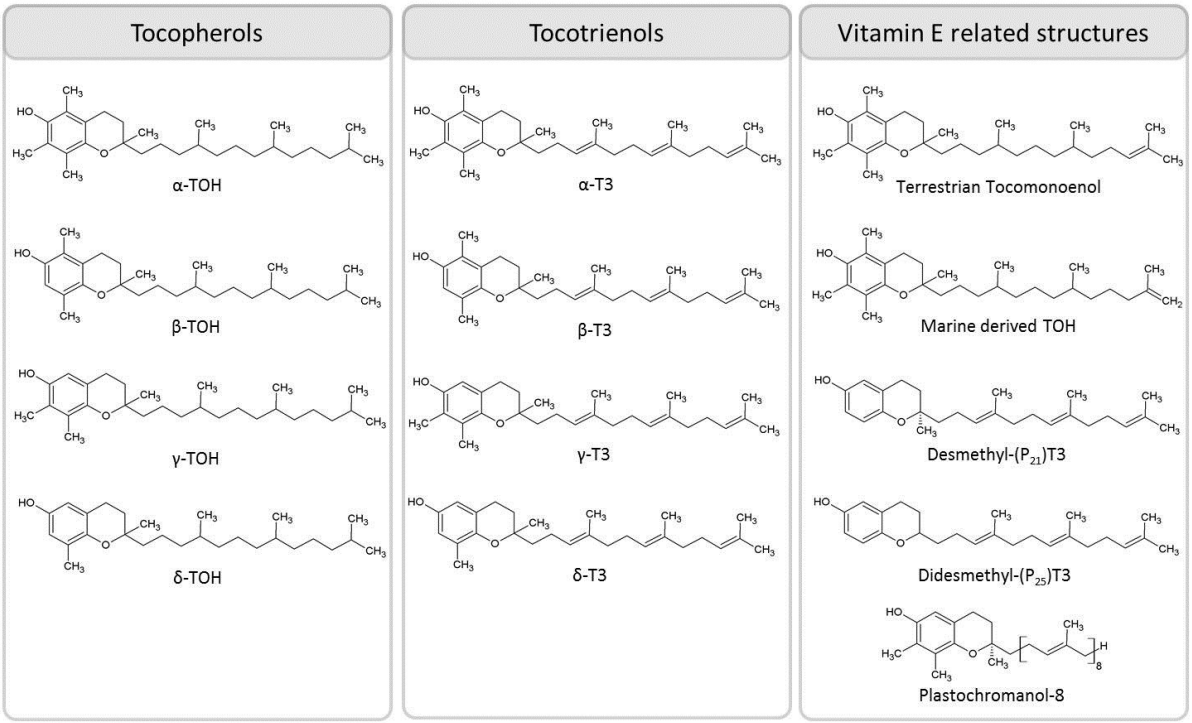

Figure 1: Structures of vitamin E components.

avocado and green olives – contain less vitamin E analogues (DUTTA & DUTTA 2003). The amount of vitamin E isomers contained in food depends on several factors such as growing, harvesting and proceeding conditions (refining, cooking) (CHUN *et al.* 2006; DUTTA & DUTTA 2003).

In detail the term vitamin E is defined as a mixture of isomers, which can be distinguished mainly into TOHs, T3s and vitamin E-related structures named tocomonoenols and marine derived TOHs (MDT) which are synthesized in cold water adapted organism (FUJISAWA *et al.* 2010) based on saturation level of the C16 side-chain (Figure 1). Tocopherols are characterized by a saturated phytyl side-chain whereas tocomonoenols, MDT and T3 are unsaturated at either the terminal isoprene unit or have three double bonds within the side-chain (KRUK *et al.* 2011; ZINGG *et al.* 2010b). Each vitamin E subgroup includes further four different isomers namely α, β, γ and δ classified depending on the methylation pattern of the hydroxychromanol ring system. Methylation may occur in either of the following configurations: (α) 5, 7, 8; (β) 5, 8; (γ) 7, 8 and (δ) 8. Tocopherol and T3 biosyntheses differ in the side-chain transferring enzymes: homogentisate (HGA)-phytyl transferase and HGA-geranylgeraniol transferase, respectively, which are responsible for the degree of saturation of the side-chain. Since HGA-phytyl transferase seems to be involved in the formation of tocomonoenols, TOHs are probably their precursors and not T3s (KRUK *et al.* 2011). The natural form of vitamin E isomers occur in *RRR* configuration, whereas the synthetic form of TOHs consists of an equal racemic mixture of the eight stereoisomers depending on the orientation of the three chiral centers: *RRR, RSR, RRS, RSS, SRR, SSR, SRS, SSS*, but there are only two possible stereoisomers for T3 isoforms (BRIGELIUS-FLOHÉ & TRABER 1999; CARDENAS & GHOSH 2013; HACQUEBARD & CARPENTIER 2005). Within the group of vitamin E *RRR*-α-TOH is assigned as the most potent member. Hence the vitamin E activities of the other isomers were calculated as effect equivalents (tocopherol equivalents, TE [%]) compared to *RRR*-α-TOH: β-TOH 50%, γ-TOH 10%, δ-TOH 3%, α-T3 30% β-T3 5% (BIESALSKI *et al.* 2002). Properties of vitamin E are related to its molecular structure which can be divided in three functionally distinct domains: the functional domain (I), the signaling domain (II), the hydrophobic domain (III), as shown in Figure 2 for α-TOH. Domains I and II are responsible for the biological activity and domain III, the side-chain, is characterized as passive domain responsible for the hydrophobicity of vitamin E and connects it thereby to lipoproteins and membranes (NEUZIL *et al.* 2002). The redox activity of domain I is related to the hydroxyl group or other substituents at position C6 at the chromanol ring. Modifications at C6, acetylation or succinylation, vary apoptotic effects of the compound (BIRRINGER *et al.* 2003). The methylation pattern on the chromanol ring comprises the second domain and is possibly involved in protein phosphatase (PP) 2A/protein kinase (PK) C signaling of vitamin E.

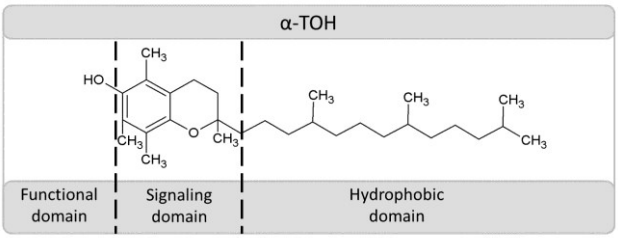

Figure 2: α-Tocopherol domains. Modified (BIRRINGER *et al.* 2003; NEUZIL *et al.* 2002)

1.1.1.1 α-Tocopherol

Until today further diverse functions of vitamin E with have been reported that are attributed to α-TOH. In early years of α-TOH research the main focus was on its radical chain breaking and radical scavenging capacity which protects cellular membranes, lipoproteins and other lipid-rich compartments (KOHLMEIER 2003) directly against lipid peroxidation. This identified α-TOH as a natural antioxidant (for further information see Chapter 1.1.4). For the first time Angelo Azzi provided evidence that next to its antioxidative capacity α-TOH has distinct properties. These so-called non-antioxidative features regulate several cell functions via signal transduction, modulation of nuclear receptors, and modulation of gene and protein expression (AZZI 2007). Based on these diverse α-TOH functions *in vitro* a role as a therapeutic agent for several serious diseases in Western industrialized countries such as diabetes, cardiovascular disease including atherosclerosis, and cancer was considered. Therefore investigations in multiple animal and human intervention and observation studies followed in the next decades; unfortunately with mostly disappointing results. Regulatory functions of α-TOH and an overview of the main important human studies are given in Manuscript II (page 42).

1.1.1.2 Tocotrienols

Next to the TOHs the four T3s are important vitamin E isomers with comparable and sometimes even stronger biological activities compared to TOHs. It was shown that T3s can alleviate α-TOH deficiency symptoms (SEN *et al.* 2007). Key functions of T3s are their antioxidative, antiatherogenic, anticancer, antidiabetic, antiinflammatory and neuroprotective properties (SEN *et al.* 2007; WONG & RADHAKRISHNAN 2012). Additionally T3-specific effects such as the suppression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity (PEARCE *et al.* 1992) which results in decreased cholesterol synthesis are known.

Apart from well known T3 isomers, α-, β-, γ-, δ-T3, Qureshi *et al.* identified two new T3 compounds 15 years ago, named desmethyl T3 [3,4-dihydro-2-methyl-2-(4,8,12-trimethyltrideca-3'(E),7'(E),11'-trienyl)-2H-1-benzopyran-6-ol] and didesmethy T3 [3,4-dihydro-2-(4,8,12-trimethyltrideca-3'(E),7'(E),11'-trienyl)-2H-1-benzopyran-6-ol]. These compounds occur in rice bran, barley and palm oil and decrease total and low density lipoprotein (LDL) cholesterol levels and inhibit HMG-CoA activity in chickens and swine (QURESHI *et al.* 2001a). Additionally they are more effective antioxidants *in vitro* and suppress B16 melanoma cell proliferation more potently than α-TOH and other T3s (QURESHI *et al.* 2000). Further, atherosclerotic plaque formation was reduced by the novel T3 compounds in C57BL/6 apolipoprotein E (ApoE)-deficient mice (QURESHI *et al.* 2001b).

1.1.1.3 Further vitamin E-related natural products

Marine derived tocopherols and α-tocomonoenol

Since MDT are predominantly present in cold-water fish to enhance antioxidant protection of cellular lipids at low temperatures. In addition, effects on membrane viscosity are considered (FUJISAWA *et al.* 2010). Precursors of α-tocomonoenol synthesis are either α-TOH or α-T3 which form α-tocomonoenol by site-specific desaturation in phytoplankton or by partial reduction during α-TOH synthesis, respectively (GOTOH *et al.* 2009), in rice bran and palm oil. The origin of MDT in fish or marine vertebrates which can accumulate in humans eating fish (YAMAMOTO *et al.* 2001) is attributed to MDT producing photosynthetic organisms, such as phytoplankton (FUJISAWA *et al.* 2010). Both α-tocomonoenol and MDT accumulate in fish liver and brain as well as in human plasma and show similar biologic availability as vitamin E. Marine derived tocopherols are partly more potent than α-TOH (FUJISAWA *et al.* 2010; YAMAMOTO *et al.* 2001).

Plastochromanol-8

Plastochromanol-8 (PC-8) is an γ-T3-related natural product which differs in the length of the side-chain from other vitamin E components; instead of three isoprene units the side-chain of PC-8 consists of eight (OLEJNIK *et al.*) (see Figure 1). It is formed out of plastoquinone-9 by tocopherol cyclase (MENE-SAFFRANE *et al.* 2010). Plastochromaol-8 occurs most abundantly in linseed oil (OLEJNIK *et al.*) and also in oils from corn seed, rape seed, soybean (SONDERGAARD & DAM 1967), maize and latex from *Havea brasilensis* (WHITTLE *et al.* 1965).

1.1.1.4 Vitamin E derivatives

Due to their accumulation *in vivo* and their better stability compared to other natural and non-natural vitamin E homologues mostly α-tocopheryl acetate (α-TA), but also phosphate (α-TP) and succinate (α-TS) (see Figure 3) are often used as supplements in animal and human trails, such as the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study. Although these molecules differ in solubility, transport, metabolism and cellular activity from α-TOH, it is stated that they may act as pro-vitamins due to their conversion to α-TOH by intestinal or epidermal esterases (ZINGG *et al.* 2010b). Zingg *et al.* give two possible explanations for the effects of synthetic derivatives (i) by directly interacting with specific proteins and cellular structures or (ii) by generally influencing organelles and membrane properties (ZINGG *et al.* 2010b).

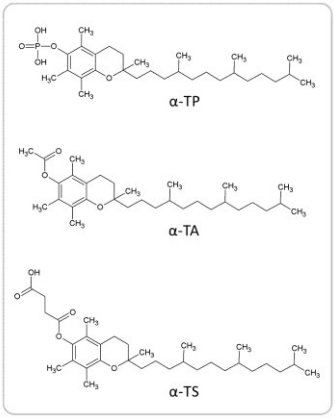

Figure 3: Tocopherol derivates.

α-Tocopheryl phosphate, for example, is discussed to act as intra- and extracellular transport, absorption and storage form of α-TOH (GIANELLO *et al.* 2005). More likely it acts as

a signal molecule representing the active form of α-TOH as a cofactor or ligand for enzymes regulating signal transduction involved in inflammatory, antioxidative, antiatherogenic (LIBINAKI *et al.* 2010) and proliferative mechanisms (NEGIS *et al.* 2007; ZINGG *et al.* 2010a). α-Tocopheryl acetate in contrast is of particular importance in skin diseases (PANIN *et al.* 2004) and seems to be potent only after its conversion to α-TOH (FIUME 2002). α-Tocopheryl succinate differs mainly from its precursor by a high selectivity for malignant cells and simultaneous lower toxicity to healthy cells possibly due to the inability of malign cells to hydrolyze esters (NEUZIL *et al.* 2002), which characterizes it as an important therapeutic agent for inhibiting cancer (DONG *et al.* 2008) and sepsis *in vivo* (CUSCHIERI *et al.* 2007).

Since the knowledge about the mechanisms and modes of action of vitamin E is incomplete the interest for natural or synthetic vitamin E isomers, derivatives and related metabolites still rises. Most of these compounds have been shown to affect pathways involved in inflammation, oxidation and of age-related diseases such as cancer, diabetes and atherosclerosis to different extent. As recently described non-α-TOH structures are partly more potent in regulation and even targets different from α-TOH-mediated effects are modulated (OLEJNIK *et al.;* ZINGG *et al.* 2010b).

1.1.1.5 Toxicology

Recent animal studies on reproductive or developmental toxic effects of natural or non-natural vitamin E components were negative but anti-mutagenic activity has been shown (FIUME 2002). Physiological vitamin E intake of 100 mg/d can be increased up to 300 mg/d without any complications (YAP *et al.* 2001; YU *et al.* 2006). Even for short-term, high-dose administration of vitamin E no adverse effects have been described. However, persistent high-dose supplementation has been shown to interfere with blood clotting and is associated therewith to increase risk of hemorrhagic stroke in animal studies (FIUME 2002). In the past TOH was considered to be a safe food additive (TOMASSI & SILANO 1986). But in the last years an increase of total mortality after high-dose vitamin E intake was discussed (MILLER 2005) (Manuscript II page 42). Excessive intake of vitamin E results in enhanced metabolism resulting in increased α-carboxyethylhydroxychromanol (α-CEHC) formation and excretion via urine and the vitamin E excretion itself via bile. This suggests that vitamin E needs to be eliminated somehow; possibly to avoid antioxidative suppression of essential reactive oxygen species (ROS) signaling pathways or interaction of vitamin E with other targets (BOUAYED & BOHN 2010).

1.1.2 Digestion

Bioavailability of vitamin E is mainly regulated via four key steps outlined here in brief:

(i) Intestinal absorption with bile and pancreas secretion and chylomicron synthesis and release,

(ii) Distribution via lipoproteins after lipolysis of chylomicrons in plasma,

(iii) Hepatic uptake and transport depending on the α-tocopherol transfer protein (α-TTP), and

(iv) Distribution and uptake in non-hepatic tissue via lipoproteins, importer and exporter proteins.

1.1.2.1 Intestinal absorption

It is known that vitamin E as a fat-soluble vitamin follows intestinal absorption, hepatic metabolism and cellular uptake processes other lipophilic and lipid molecules (RIGOTTI 2007). The absorption rate of vitamin E varies interindividually between 20-80% (BJØRNEBOE *et al.* 1990; RIGOTTI 2007), and is thus much lower than for other fat-soluble vitamins such as vitamin A (BOREL *et al.* 2001). The reasons for individual differences in absorption rate are diverse. Increased administration of α-TOH (TRABER *et al.* 1986) and parallel intake of additional food ingredients decrease non-α-TOH and α-TOH absorption such as retinoic acid (BIERI *et al.* 1981), plant sterols (RICHELLE *et al.* 2004), eicosapentaenoic acid, chronic alcohol consumption (BJØRNEBOE *et al.* 1990), and dietary fiber (DOI *et al.* 1983). Apparently crucial for vitamin E absorption is the way how it is presented to the intestinal surface (SHILS *et al.*). Additionally the supplied form of α-TOH, as free molecule or e.g. as α-TA, a common food additive, is of particular importance for its bioavailability (BAKER *et al.* 1980; BURTON *et al.* 1988). Lipids and fat-soluble compounds are processed enzymatically in the stomach by lipase first. Vitamin E occurs in human diet mainly as free α-TOH, therefore the importance of the degradation in the stomach is probably limited (BOREL *et al.* 2001). The need of such gastric degradation for stabilized forms of vitamin E such as α-TA is not known due to a lack of investigations (BOREL *et al.* 2001). The more stable α-TA, a common food additive, however needs further hydrolysis directly by the bile acid-dependent lipases in the pancreas or an intestinal mucosal esterase (BJØRNEBOE *et al.* 1990). Subsequent vitamin E absorption in the duodenum is characterized by the transfer from the emulsion fat globules to water-soluble multi- and unilamellar vesicles and mixed micelles made of phospholipids and bile salts, the so-called lipid emulsification. This is the fundamental step in lipid digestion which is therefore the crucial step in vitamin E absorption (see Figure 4). Since the transport of vitamin E to enterocytes is less efficient compared to other types of lipids. This may explain the low vitamin E bioavailability (BOREL *et al.* 2001). The intestinal cellular uptake of vitamin E from mixed micelles follows two different pathways across enterocytes as it has been shown *in vitro* and *in vivo*: via (i) passive diffusion and (ii) scavenger receptor class B type 1 (SR-B1) (REBOUL *et al.* 2006). Additionally, the Niemann-Pick C1-like protein 1 (NPC1L1), an apical

membrane receptor of the small intestine (HACQUEBARD & CARPENTIER 2005), transports α-TOH across the enterocyte membrane (NARUSHIMA *et al.* 2008). Niemann-Pick C1-like protein 1 is further responsible for the reuptake of intact α-TOH from bile to hepatocytes and prevents the excessive biliary loss of α-TOH together with α-TTP, which is responsible for the storage of α-TOH in the liver (NARUSHIMA *et al.* 2008). Very important ATP-binding cassette (ABC) transporters are ABCG5/ABCG8 and ABCA1/ABCG1, which are responsible for steroid efflux into the intestinal lumen and transport into the lymph system, respectively. Vitamin E up-regulates these transporters in rats which once shows the contribution of vitamin E on cholesterol absorption and secretion (ROGI *et al.* 2011). Finally, ABCA1 is directly involved in vitamin E export (LEMAIRE-EWING *et al.* 2010).

Until now no intestinal absorption discrimination of *RRR*-α-TOH over other stereoisomers (KIYOSE *et al.* 1995) or rather γ-TOH (TRABER & KAYDEN 1989) is known. Absorption efficiency and integration of α-TOH and γ-TOH into chylomicrons are equal.

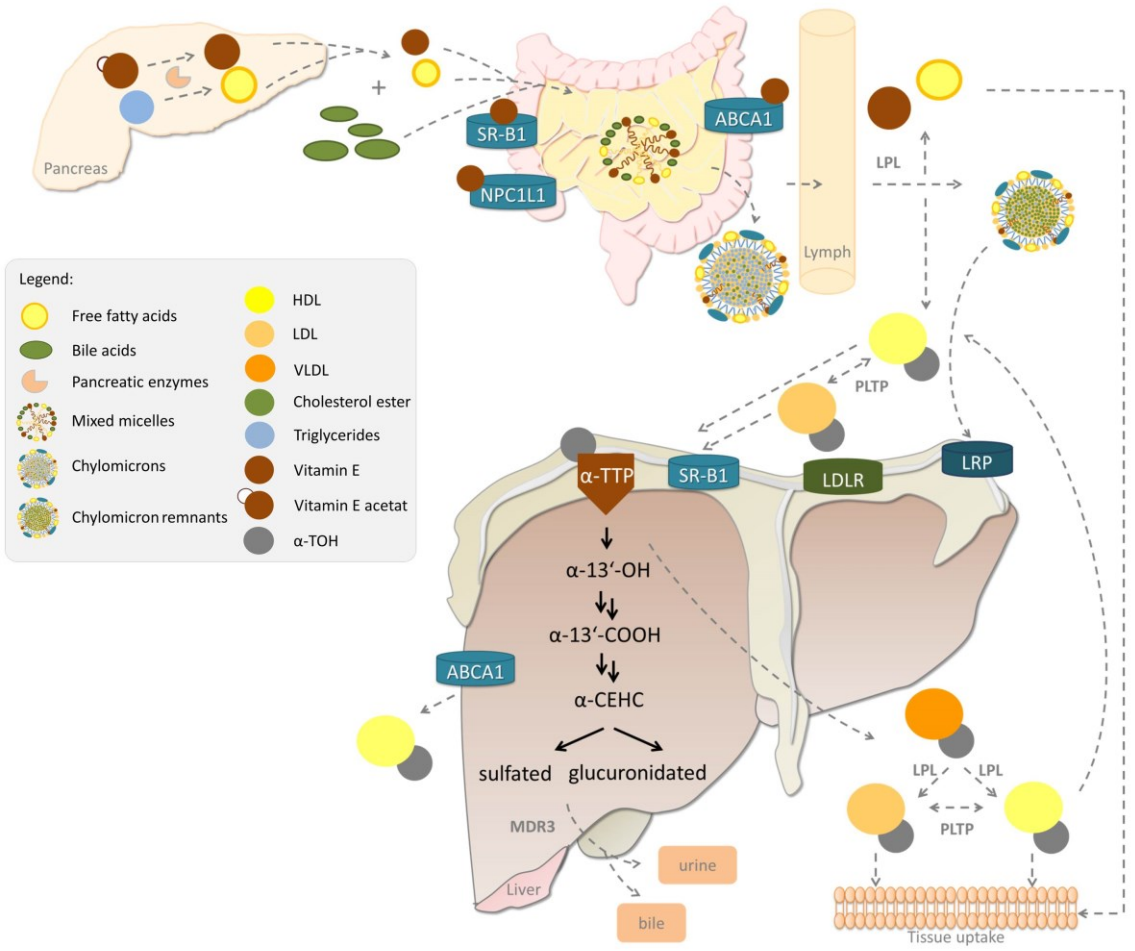

Figure 4: Digestion, transport and degradation of vitamin E. Course of vitamin E after supplementary or nutritional intake follows in general the way of further coexisting lipid compounds. Pancreatic and intestinal enzymatic digestion followed by the circulation and distribution to the liver and non-hepatic tissues is the same for all vitamin E isomers. Discrimination in favor of α-TOH occurs mainly in the liver by the α-TTP, protecting α-TOH from excessive degradation and excretion. (BJØRNEBOE *et al.* 1990; BOREL *et al.* 2001; HACQUEBARD & CARPENTIER 2005; LEMAIRE-EWING *et al.* 2010)

1.1.2.2 Transport of vitamin E in plasma

The transport of vitamin E in the blood follows largely the lipoprotein metabolism. Chylomicrons containing vitamin E, independent from the isomers or stereoisomers, and are secreted into the intestinal lymph system starting in the systemic circulation. Chylomicrons are catabolized via lipoprotein lipase (LPL) (HACQUEBARD & CARPENTIER 2005; KIYOSE *et al.* 1997). During this lipolysis step vitamin E is either incorporated into high density lipoprotein (HDL) and to other circulating lipoproteins such as LDL by the phospholipid transfer protein (PLTP) (SHILS *et al.*), or to chylomicron remnants. An additional member of the lipid transfer proteins, the cholesteryl ester transfer protein (CETP), is discussed to take part in vitamin E transport and metabolism (LEMAIRE-EWING *et al.* 2010). Vitamin E incorporated in chylomicron remnants is further imported into the liver via LDL receptor-related protein (LRP) and the LDL receptor (LDLR) (HERZ *et al.* 1995; LEMAIRE-EWING *et al.* 2010). Until now no specific plasma transport proteins for α-TOH have been described (BJØRNEBOE *et al.* 1990; TRABER 2013). Lipoproteins serve as carriers for lipophilic molecules, such as vitamin E, to transport them between lipoproteins and from lipoprotein to tissue (BJØRNEBOE *et al.* 1990). Under physiological conditions α-TOH is mostly transported via very low density lipoprotein (VLDL) and HDL (BJØRNEBOE *et al.* 1986), whereas under fasting conditions LDL take on this task. Although VLDL have the highest capacity to carry α-TOH, these particle represent the smallest fraction in vitamin E transport via lipoprotein in the circulation in the fasted state (BEHRENS *et al.* 1982).

1.1.2.3 Hepatic transport

Studies in rats have shown that α-TOH is present in the liver and plasma after one week of supplementation, but in adipose tissue only after two months of supplementation (WEISER *et al.* 1996). α-Tocopherol is secreted by the liver in nascent lipoproteins such as VLDL whereas γ-TOH is secreted to bile (TRABER & KAYDEN 1989). Hence discrimination of TOH isomers and stereoisomers occurs mostly in the liver via α-TTP which discriminates predominantly in favor of α-TOH and preferentially secretes isomers with 2*R* configuration (*RRS, RSR, RSS*) and *RRR* configuration to VLDL. α-Tocopherol from the plasma accumulates in adipose tissue, liver and brain (but also 2*S* isomers are present in these tissues) (KIYOSE *et al.* 1997; WEISER *et al.* 1992). The lipoproteins LDL, VLDL and HDL preferentially take up *R*-isomers instead of *S*-isomers, therefore the bioavailability of *RRR*-α-TOH is significantly greater than that of all/-rac-α-TA (KIYOSE *et al.* 1997). Traber *et al.* mentioned that lipoproteins returned *RRR*-α-TOH to the plasma in unaltered form, whereas all non-*RRR*-α-TOH are metabolically degraded primarily followed by their excretion (GIANELLO *et al.* 2005; TRABER *et al.* 1990). In the liver of rats, parenchymal cells, such as Kupffer cells, store 75-90% of injected α-TOH which is suggested to be predominantly located in mitochondria, lysosomes and Golgi apparatus compared to non-parenchymal cells (BJØRNEBOE *et al.* 1990). Arita and co-workers showed that α-TOH secretion out of the liver is independent of VLDL assembly and secretion and suggested that oxysterol-binding proteins (ARITA *et al.* 1997) or ABCA1 (ORAM *et al.* 2001) are possibly involved in α-TOH secretion. Finally, only 1% of the

total body TOH is transported in the blood (HORWITT *et al.* 1972).

1.1.2.4 Intercellular transport and intracellular distribution of vitamin E – tocopherol binding proteins

Both SR-B1 and ABCA1 are of particular importance for the uptake and efflux within the vitamin E distribution pathways (LEMAIRE-EWING *et al.* 2010). Scavenger receptor B1 mediates vitamin E uptake in the intestine and in peripheral tissue whereas LDL and LRP are involved in uptake in the liver. Efflux of vitamin E via ABCA1 takes place in the intestine and the liver. Additionally, ACBA1 regulates the cellular efflux of vitamin E in macrophages and fibroblasts (ORAM *et al.* 2001). This finding reinforces the presumption of similarities between cholesterol and α-TOH transport. Further the multidrug resistance (MDR) P glycoprotein (Pgp or MDR1) has been identified to regulate α-TOH transport across the plasma membrane and its secretion into the bile (MUSTACICH *et al.* 1998).

Next to α-TTP further intracellular α-TOH transporters, the tocopherol associated protein (TAP) and the tocopherol-binding protein (TBP), are known. Tocopherol associated protein as a member of the hydrophobic lipid-binding protein family seems to be identical with the supernatant protein factor (SPF) (STOCKER *et al.* 1999), and mediate antiproliferative effects in different prostate cancer cells by promoting vitamin E uptake or independently of α-TOH effects (CARDENAS & GHOSH 2013; NI *et al.* 2005), whereas TBP may be involved in intracellular distribution of α-TOH (HACQUEBARD & CARPENTIER 2005). Supernatant protein factor is involved in cholesterol synthesis, whereas TAP/SPF forms complexes with *RRR*-α-tocopheryl quinone, the oxidation product of α-TOH, but the function of SPF needs further investigation (SHILS *et al.;* STOCKER & BAUMANN 2003). Both α-TTP and TAP belong to a family of ligand binding proteins that have a *cis*-retinal binding motif sequence, the so-called CRAL_TRIO. All members of this family bind α-TOH but in a minor proportion compared to α-TTP, but their physiological relevance is unknown (SHILS *et al.*). Further an α-TOH binding protein of 14.2 kDa has been detected in the liver which is characterized by similar specificity for α-TOH compared to α-TTP and is important for the regulation of cellular α-TOH concentration and intracellular α-TOH transport (DUTTA-ROY *et al.* 1993). As α-TTP occurs additionally in placenta the importance of α-TOH to prevent fetus resorption is obvious (TRABER & SIES 1996) and the expression of α-TTP in several other tissues suggests relevance for intra-organ traffic (BRIGELIUS-FLOHÉ 2009).

1.1.3 Metabolism of vitamin E

In contrast to other fat-soluble vitamins vitamin E does not accumulate in the liver to toxic levels (SHILS *et al.*), because excessive vitamin E intake leads to increased vitamin E metabolism. Within the group of vitamin E, α-TOH is dominant in animals and humans, as a result of the postulated α-TTP discrimination or rather enhanced metabolic degradation of the non-α-TOH isomers by cytochrome P450 enzymes and their subsequent elimination via

urine (ZINGG *et al.* 2010b). As already mentioned hepatic α-TTP preferentially promotes the incorporation of α-TOH in VLDL in the liver. Hosomi and co-workers estimated the relative affinities of α-TTP to different vitamin isomers and stereomers starting from *RRR*-α-TOH set to 100%: β-TOH 38%, α-T3 12%, *SRR*-α-TOH 11%, γ-TOH and trolox 9% followed by δ-TOH, α-TA and α-TOH quinone with 2%, (HOSOMI *et al.* 1997). Requirements for binding of vitamin E isomers and derivates to α-TTP are the three methyl groups of the chromanol ring system, especially at position C5, the free hydroxyl group and the phythyl side-chain (HOSOMI *et al.* 1997). Since the affinity of vitamin E isomers to α-TTP reflects the biological activity from rat resorption-gestations assay (LETH & SONDERGAARD 1977), the hypothesis that α-TTP is responsible for the discrimination of α-TOH is supported. A current study from Grebenstein *et al.* raises the presumption that the metabolism of vitamin E but not α-TTP discriminates mainly non-α-TOH isomers, as α-TTP protects the side-chain of vitamin E isomers from ω-hydroxylase-induced degradation (GREBENSTEIN *et al.* 2014). High levels of expression of α-TTP lead to higher intracellular concentrations of γ-TOH combined with a reduced production of γ-CEHC, which confirms the concept that α-TTP binding protects from degradation. As suggested by Grebenstein *et al.* the long-chain metabolites (LCMs) of vitamin E are possible ligands for TTP and/or other hepatic TOH binding proteins. Further, the importance of these proteins for LCM transfer to mitochondria needs to be investigated (GREBENSTEIN *et al.* 2014).

In the liver the metabolism of vitamin E, mainly α-TOH, occurs in three cell compartments: microsomes, peroxisomes and mitochondria (Figure 5). So far it is not known how the metabolites enter the peroxisome or leave it. In microsomes side-chain truncation is initiated by CYP4F2/3A4-dependent ω-hydroxylation which is the initial step resulting in the formation of the alcohol derivate α-13'-OH (13'-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyltridecanol) (PARKER *et al.* 2000; SONTAG & PARKER 2002). Subsequent α-oxidation in peroxisomes forms the acid derivate α-13'-COOH (13'-(6-

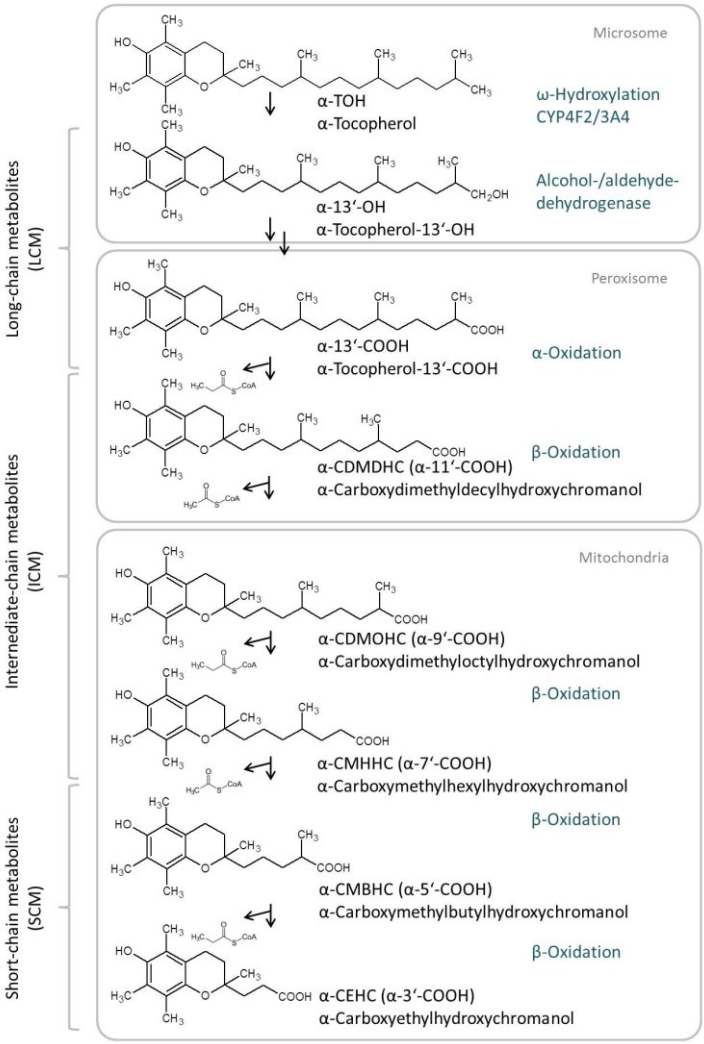

Figure 5: Physiological hepatic metabolism of α-TOH. Modified from (BIRRINGER *et al.* 2002a; MUSTACICH *et al.* 2010)

hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyltridecanoic acid) via the aldehyde metabolite processed by alcohol and aldehyde dehydrogenase activity. The hydroxychromanols and carboxychromanols, will be abbreviated in the presented manuscript as α-13'-OH and α-13'-COOH, respectively and classified as LCMs. In the peroxisomes two β-oxidation cycles cause the elimination of either propionyl-CoA or acetyl-CoA and formation of the intermediate-chain metabolites (ICM), followed by three cycles of mitochondrial β-oxidation. This results first in short-chain metabolites (SCM) and finally in α-CEHC, the catabolic end-product of TOH and T3 degradation (BIRRINGER *et al.* 2002a). The CEHC precursor carboxymethylbutylhydroxychromane (CMBHC) is the main product of T3 metabolism *in vitro* which may be explained by a slower CMBHC to CEHC conversion compared to the previous β-oxidation steps (BIRRINGER *et al.* 2002a). Until now the CEHC isomers α-, δ- and γ-CEHC have been detected in sulfated or glucuronidated form (CHIKU *et al.* 1984; SWANSON *et al.* 1999) and are further excreted via urine (BRIGELIUS-FLOHÉ & TRABER 1999) or bile (KIYOSE *et al.* 2001). But these metabolites were also found in plasma (STAHL *et al.* 1999). Although vitamin E catabolism is more rapidly for T3s than TOHs and varies within the TOHs, the metabolic pathway is the same (BIRRINGER *et al.* 2002a). In contrast to TOHs the double bonds of T3 remains in the side chain while entering the β-oxidation pathway which implies the involvement of further enzymes, possibly two auxiliary enzymes 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase known from linoleic acid metabolism (BIRRINGER *et al.* 2002a). γ-Tocopherol is metabolized faster than α-TOH which can be explained partly by a higher affinity of the ω-hydroxylase to γ-TOH than α-TOH (SONTAG & PARKER 2002). The SCM of γ-TOH γ-CEHC is excreted via urine and bile (KIYOSE *et al.* 2001). Parallel administration of α-TOH and γ-TOH in equal amounts leads to increased γ-CEHC excretion via bile (6 h to 18 h) and urine (18 h to 36 h) compared to sole administration of γ-TOH (KIYOSE *et al.* 2001). The authors concluded that γ-CEHC excretion is gradually shifted from bile to urine and that α-TOH may affect γ-TOH metabolism (KIYOSE *et al.* 2001). Only 1-3% of the intaken *RRR*-α-TOH was catabolized to α-CEHC (SCHUELKE *et al.* 2000). Supplementation of α-TOH led simultaneously to increased α-TOH plasma levels and higher amounts of α-CEHC in urine. Hence α-CEHC can be used as a marker for α-TOH status in healthy humans (SCHULTZ *et al.* 1995). Schuelke and co-workers, however, suggest that α-CEHC excretion may increase only after exceeding an individual α-TOH threshold (30-50 µM), depending on plasma lipid concentrations (HORWITT *et al.* 1972; SCHULTZ *et al.* 1995); and is therefore a marker for (super)optimal α-TOH supply (SCHUELKE *et al.* 2000). Schultz *et al.* first identified α-CEHC as the metabolic end-product of α-TOH in human urine and called into question the hitherto so-called Simon metabolites. They suggested that the Simon metabolites are oxidative workup artifacts (SCHULTZ *et al.* 1995) (see Chapter 1.1.4.1). Next to the established α-carboxyethylhydroxychromanol, α-CEHC glycine, α-CEHC glycine glucuronide and α-CEHC taurine have been detected in humans and mice by Johnson suggesting that further metabolic pathways for vitamin E may exist (JOHNSON *et al.* 2012).

Vitamin E metabolism is not specific. Since hepatic metabolism of vitamin E follows xenobiotic pathways, xenobiotic transporters may act as transporters for the metabolic end-

product CEHC (SHILS *et al.*). Cometabolism of xenobiotics and vitamin E may affect bioavailability and mechanisms of regulation by using the same transporters and enzymes (JOHNSON *et al.* 2012), as described for γ-TOH and simvastatin (TRABER 2010). As the pregnane X receptor (PXR) binds vitamin E and regulates xenobiotic detoxification via CYP3A it may be important for vitamin E metabolism and regulation of its xenobiotic pathways (TRABER 2010). The second cytochrome P450 member CYP4F2, which mediates ω-hydroxylation is not specific for vitamin E and plays also a role in eicosanoid and vitamin K_1 metabolism (TRABER 2010), which represents the diversity and thus the complexity of vitamin E metabolism.

1.1.3.1 Vitamin E deficiency and metabolic disorders

As vitamin E is primarily stored to a large extent in adipose tissue (90%, SHILS *et al.*) vitamin E deficiency is almost unknown under physiological conditions. Symptoms of slight vitamin E undersupply become apparent late, usually after decades (SHILS *et al.*), since vitamin E can be mobilized from adipose tissue storages. In contrast, serious vitamin E deficiencies manifest in acute symptoms, such as neuropathy and myopathy, as vitamin E is essential for the development of the central nervous system. Next to already described dietary habits several hereditary disorders are known which result in inadequate vitamin E bioavailability and deficiency, such as malabsorption (SHILS *et al.*), *abetalipoproteinaemia*, caused by a mutation in the microsomal triglyceride transfer protein, as well as, the familial isolated vitamin E deficiency, which is also known as *ataxia with isolated vitamin E deficiency* (AVED) and is caused by various mutations concerning the α-TTP resulting in inadequate distribution of vitamin E in the periphery (OUAHCHI *et al.* 1995; SCHUELKE *et al.* 2000). Further *Marinesco-Sjögren syndrome* and chylomicron retention disease known as *Anderson* disease are possible reasons for severe vitamin E deficiency. Both diseases are caused by a failure of enterocytes to assemble or deliver chylomicrons due to disturbed intestinal fat transport (AGUGLIA *et al.* 2000; GORDON 2001).

1.1.4 Vitamin E – antioxidative or prooxidative capacity

1.1.4.1 Antioxidant capacity

Within the group of vitamin E α-TOH is known as the most important lipid-soluble radical chain-breaking anti-oxidant, mainly due to its protection against peroxidation of PUFA in phospholipids in cell membranes and plasma lipoproteins at least *in vitro* (Figure 6) (BRIGELIUS-FLOHÉ 2009; BURTON & INGOLD 1989). Higher PUFA intake requires a higher vitamin E

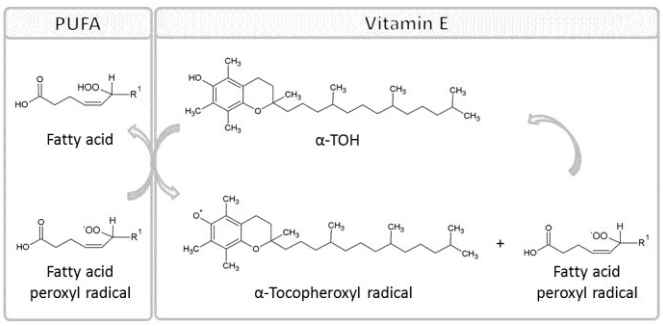

Figure 6: Mechanisms of classical antioxidant activity under strong oxidative conditions. (BJØRNEBOE *et al.* 1990; BRIGELIUS-FLOHÉ 2009; KONTUSH *et al.* 1996)

supply to provide adequate antioxidative capacity against lipid peroxidation. Fatty acids due to their sterical properties tend to form radicals which can be scavenged by the free hydroxyl group at the chromanol ring of α-TOH. To recover the intact α-TOH molecule it reacts with a second peroxyl radical or tocopheroxyl radical under strong oxidative conditions. Additionally, oxidation of α-TOH in the liver forms α-TOH quinone and further α-TOH hydroquinone which is excreted to the bile after conjugation with glucuronic acid (BJØRNEBOE *et al.* 1990). Subsequently tocopheronic acid and tocopherono-lactone arise and are excreted via the second possible pathway, the urine (BJØRNEBOE *et al.* 1990). These metabolic products first were described by Simon *et al.* in 1956, and are therefore called Simon metabolites. They were probably wrongly accepted as the catabolic end-products of vitamin E metabolism instead of CEHC for many years (see Figure 7) (SCHULTZ *et al.* 1995). Nevertheless formation of Simon metabolites indicates that antioxidant reactions of α-TOH cannot be excluded completely (BRIGELIUS-FLOHÉ 2009).

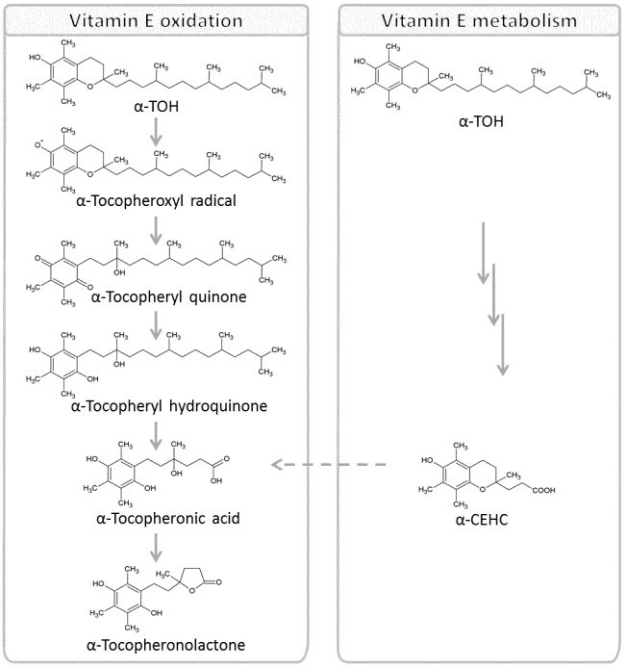

Figure 7: Formation of Simon metabolites. Modified from (SCHULTZ *et al.* 1995)

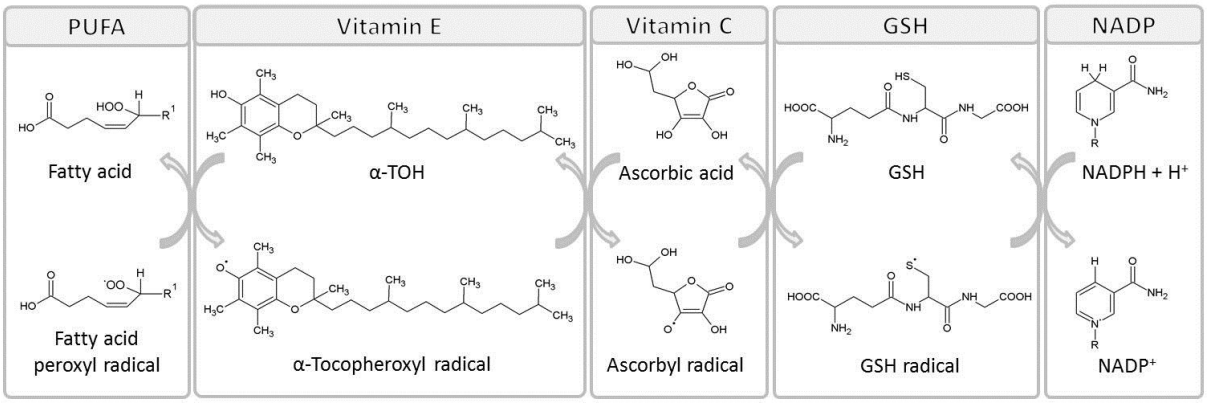

Figure 8: Antioxidant activity under mild oxidative conditions (KONTUSH *et al.* 1996).

Under mild oxidative condition the tocopheroxyl radical needs coantioxidants to be regenerated into its intact TOH form which can be used for scavenging free radicals in a hydrophobic milieu (see Figure 8) (KONTUSH *et al.* 1996). The most important coantioxidant is ascorbic acid which further forms an ascorbyl radical under regeneration of α-TOH. The reduction to ascorbic acid transfers the radical to glutathione (GSH). The subsequent reduction back to GSH is enabled by nicotinamide adenine dinucleotide phosphate (NADPH) providing its hydrogen.

1.1.4.2 Vitamin E acting as a prooxidant

Next to the extensively investigated antioxidative properties of vitamin E/α-TOH prooxidative properties of high vitamin E doses *in vitro* and *in vivo* are discussed. High levels of cations such as ferrous ions in combination with α-TOH *in vitro* could increase production of hydroxyl radicals or reactive proteins bound to α-TOH at hydrophilic membrane sites (SCHWARTZ 1996). Even *in vivo* vitamin E supplementation was described repeatedly as a potential risk factor for increasing gastrointestinal symptoms and total mortality (MILLER 2005), possibly induced by increased oxidative activity in plasma (PEARSON *et al.* 2006). Apart from the question if vitamin E acts as a pro- or antioxidant several studies simply showed no effect after supplementation whether at high or low doses (WALLERT *et al.* 2014). As investigated by Kontush and coworkers the availability of α-TOH-related coantioxidants, such as ascorbic acid or bilirubin, determines the behavior of α-TOH as pro- or antioxidant under mild oxidative conditions in human plasma or isolated LDL particles (KONTUSH *et al.* 1996), whereas under a deficiency of coantioxidants α-TOH exerts its prooxidant properties. However, under strong antioxidative conditions α-TOH acts independently of coantioxidants. Increased oxidation under mild oxidative conditions may be caused a slight parallel increase of PUFAs and is reversible by adding physiological amounts of ascorbic acid as observed by Kontush (KONTUSH *et al.* 1996). In contrast to these results observations from other groups do not confirm the prooxidative effects of α-TOH *in vivo* (ROBERTS *et al.* 2007). α-Tocopherol is quantitatively the most important antioxidant occuring in LDL particles, next to γ-TOH, carotenoids and ubiquinol-10 (KONTUSH *et al.* 1996). Since oxidation of LDL is a key event in atherogenesis α-TOH may play an important role in this initial step.

1.1.4.3 Vitamin E in biological membranes

Vitamin E isomers are components cellular of membranes with the highest accumulation in liposome membranes; more precisely vitamin E is located in the phospholipid bilayer in different conformations depending on the isomer class (Figure 9). In contrast to TOHs containing three chiral centers T3s have only one which explains the different conformations, position in and penetration of phospholipid bilayers of cellular membranes (Figure 9) (ATKINSON *et al.* 2010; SEN *et al.* 2007; WONG & RADHAKRISHNAN 2012). Crucial for incorporation into membranes are the methylation and the saturation patterns of vitamin E isomers which determine

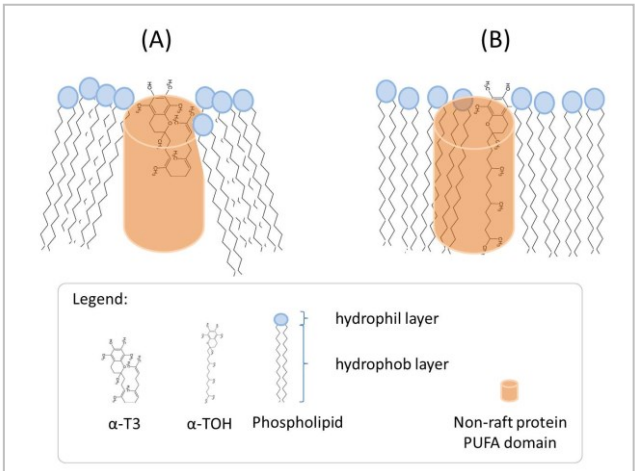

Figure 9: Steric disturbance of phospholipids by α-T3 (A) and α-TOH (B) in biological membranes near non-raft domains (ATKINSON *et al.* 2010; SEN *et al.* 2007).

their lipophilicity (BRIGELIUS-FLOHÉ 2009). In general, α-TOH occurs in membranes 1:100-1000 compared to phospholipids (BRIGELIUS-FLOHÉ 2009).

The structural role of α-TOH in lipid domains of all membranes may be analogous to that of cholesterol in lipid rafts (ATKINSON *et al.* 2010; MARQUARDT *et al.* 2013). But discrepancies occur about the precise localization of α-TOH within the phospholipid bilayer. α-Tocopherol can segregate in lipid rafts (ROYER *et al.* 2009), but its localization in PUFA-rich domains seems to be more likely (ATKINSON *et al.* 2010). This so-called lipid raft hypothesis can be one possible regulatory pathway of α-TOH in signal transduction, possibly by modifying protein-lipid and protein-protein interactions, because most of the enzymes known to be regulated by α-TOH are associated with lipid rafts (LEMAIRE-EWING *et al.* 2010).

Jeffrey Atkinson mentioned that the membrane saturation status is apparently responsible for the depth the hydroxyl group of α-TOH penetrates in the membrane (ATKINSON *et al.* 2010) which affects its antioxidative abilities. This hypothesis is questioned by findings of Marquardt *et al.* who did not find an association between hydrocarbon saturation and α-TOH location (MARQUARDT *et al.* 2013). Further different hypotheses have been proposed on how deep α-TOH penetrates in the membrane (AFRI *et al.* 2004). In general the chromanol ring is accepted as the outer part of the vertically arranged α-TOH either located deep in the phospholipid bilayer or rather at the hydrophobic-hydrophilic interface. The localization of α-TOH with the C5 and C7 methyl groups near the interface makes it possible to inhibit lipid oxidation more efficiently (AFRI *et al.* 2004; MARQUARDT *et al.* 2013) and to interact with ascorbic acid for subsequent regeneration of α-TOH after radical reaction. The antioxidative capacity of α-TOH does not prevent radical formation in the cytosol or even initial oxidation of fatty acyl chains but it probably intercepts free radicals near the lipid/water interface and terminates lipid peroxide chain reactions deep in the membrane (MARQUARDT *et al.* 2013). Diplock and coworkers proposed that the antioxidative capacity of α-TOH on PUFAs is explained by the formation of complexes of α-TOH and non-raft PUFA chains of membrane phospholipids, whereas pockets are formed by the double bonds of the PUFA and the methyl groups at positions C4 and C8 of the isoprenoid side-chain of α-TOH (DIPLOCK & LUCY 1973).

Furthermore, to protect lipid membrane components against lipid peroxidation, α-TOH stabilizes membranes as a result of decreasing its fluidity possibly by forming complexes with destabilizing lipid compounds, such as free fatty acids or lysophospholipids within the membrane (BRIGELIUS-FLOHÉ 2009; LEMAIRE-EWING *et al.* 2010). α-Tocopherol may be involved in signaling and membrane processes, such as fusion, vesicular transport, and transmitter release (BRIGELIUS-FLOHÉ 2009), maybe via specific α-TOH membrane receptors (ZINGG *et al.* 2010a).

1.1.5 Non-antioxidative properties of vitamin E

Since vitamin E scavenges ROS and nitrogen species function of vitamin E was believed to be limited to its antioxidant properties for several years (ZINGG *et al.* 2010a). Properties

independent of the antioxidation were first suggested in the early fifties but not pursued for further decades (ABE *et al.* 2007; AZZI 2007). Research of Angelo Azzi and colleagues over years brought forth several working hypotheses regarding non-antioxidative properties of vitamin E or rather α-TOH. Thereby the focus of vitamin E research changed towards the modulation of gene regulation and enzyme activities, signaling cascades within uptake, transport, degradation, metabolism and excretion of TOH, lipoprotein uptake and inflammation, to name only a few (BRIGELIUS-FLOHÉ 2009). These non-antioxidative properties control also severally events in atherosclerosis, for example inhibition of smooth muscle cell (SMC) proliferation, preservation of endothelial integrity, inhibition of monocyte-endothelial adhesion, inhibition of monocyte ROS and cytokine release, and inhibition of platelet adhesion and aggregation (AZZI 2007). The initial finding in this field was the inhibition of PKC activity and vascular SMC growth by α-TOH (AZZI *et al.* 2002). Following observations demonstrated the inhibition of PKC in various cell types with consequent inhibition of platelet aggregation, nitric oxide production in endothelial cells (EC) and superoxide production in neutrophils and macrophages (AZZI *et al.* 2002). In addition, scavenger receptors (SR), adhesion molecules and collagenase seem to be under the non-antioxidant control of α-TOH (AZZI *et al.* 2002). In summary, the spectrum of action includes reactions on the level of cellular processes and transcriptional regulation (AZZI *et al.* 2002). A detailed overview of non-antioxidative regulation by α-TOH presented in Manuscript II (see page 42).

Antioxidative properties were confirmed *in vitro* but validation mostly failed *in vivo* when accepted markers of oxidative damage were considered (BRIGELIUS-FLOHÉ 2009). Non-α-TOH isomers known for their smaller antioxidative capacity show comparable cellular functions which suggest that molecular structures are responsible for providing non-antioxidant functions (AZZI 2007). Finally the identification of a key regulator by which α-TOH influences gene activity is still missing (BRIGELIUS-FLOHÉ 2009).

1.1.6 Prevention of age-related diseases by vitamin E

In the USA about 11% of the population consumes vitamin E supplements daily (PEARSON *et al.* 2006). Also in Germany vitamin E supplementation is widespread although the effects of vitamin E are only incompletely understood. In recent years the discussion whether vitamin E increases all-cause mortality was sparked by Miller (MILLER 2005), but the question has not been resolved yet (TRABER 2007). The Heart Outcomes Prevention Evaluation Study Extension (HOPE-TOO) trial reported a higher risk of heart failure and hospitalization due to heart failure in cardiovascular disease patients after vitamin E supplementation. But until today evidence for adverse side effects of vitamin E supplements up to 1000 mg (1600 IU) in healthy people are missing (HATHCOCK *et al.* 2005). On the other hand the benefit of vitamin E or rather α-TOH on age-related diseases, such as cardiovascular disease (CVD), cancer or Alzheimer disease, is still not proven (FORTMANN *et al.* 2013). Several antitumor effects of vitamin E have been shown *in vitro* and in animal studies but failed to be reproduced in humans (WADA 2012). In contrast to TOH the T3 are a further option for intervention in cancer studies but large-scale clinical trials are still missing (WADA

2012). In addition to unconfirmed anticancer effects, possibly cancer promoting effects have been shown in the Selenium and Vitamin E Cancer Prevention Trial (SELECT) which focused on the impact of vitamin E supplementation and the development of prostate cancer (KLEIN & THOMPSON 2012). Similar observations were made for CVD and vitamin E supplementation (DUTTA & DUTTA 2003). As discussed in several reviews the promising *in vitro* reports on vitamin E acting as an antioxidant failed to be reproduced *in vivo* (CORDERO *et al.* 2010). As evidence supporting the hypothesis that α-TOH does not act as an antioxidant in human atherosclerotic plaques, coexistence of oxidized lipids and α-TOH has been suggested (SUARNA *et al.* 1995). For further details regarding benefits of vitamin E supplementation on CVD see also Manuscript II within this thesis (page 42). Similar observations have been made also for other antioxidants.

Alzheimer disease is discussed to be at least partly a vascular disease. Since oxidative processes are leading cause of Alzheimer disease, treatment with antioxidants such as vitamin E is possibly relevant in disease prevention. Except for one clinical trial (SANO *et al.* 1997), no clinical benefit of vitamin E supplementation has been described, although decreased lipid peroxidation in plasma and cerebrospinal fluid has been found (ARLT *et al.* 2012). As known a positive correlation of vitamin E supplementation and CVD in intervention trials failed for several reasons. Comparable observations have been described for vitamin E treatment and prevention of Alzheimer disease (BREWER 2010). Therefore Farina *et al.* concluded that vitamin E supplementation for protecting against Alzheimer disease is questionable (FARINA *et al.* 2012). Further investigations are needed to clarify the mode of action of vitamin E in preventing Alzheimer disease with special focus on individual changes in redox potential (BREWER 2010; LLORET *et al.* 2009).

Angelo Azzi provided further data on *in vitro* antioxidants, e.g. which do not have comparable effects *in vivo* and concluded that chemically tested antioxidants do not have necessarily antioxidant properties *in vivo* (AZZI 2007).

1.1.7 Physiological metabolites of α-TOH

1.1.7.1 Detection of physiological metabolites

Vitamin E metabolism has been studied intensively since the 1990`s but is not yet complete. The current knowledge on vitamin E metabolism is summarized in section 1.1.3. Starting in hepatic cell culture systems first analyses regarding formation of different TOH and T3 metabolites were performed (BIRRINGER *et al.* 2002a; SONTAG & PARKER 2002). For many years detection of the catabolic end-product of vitamin E metabolism CEHC, mainly γ-CEHC and α-CEHC, has been known. These so-called SCM are commonly found in rodent and human urine (CHO *et al.* 2009; LEBOLD *et al.* 2012) as well as human plasma (HENSLEY *et al.* 2004). Zhao and coworkers detected successfully different TOH and T3 metabolites in mice (feces, urine, liver and serum) and in human (feces, urine and serum) samples (ZHAO *et al.* 2010). They were able to detect unconjugated SCMs, intermediate metabolites and LCMs of α-, γ-, δ-TOH

and their precursor, TOHs and T3s themselves, in fecal samples of mixed-TOH supplemented mice and TOH supplemented humans. Since α-TOH is primarily taken up via α-TTP and thus escapes the degradation, as described previously, the amounts of non-α-TOH metabolites are increased compared to α-TOH metabolites (ZHAO *et al.* 2010). In murine and human urine samples SCM of TOH and T3 have been detected only as glucuronide conjugates. In serum and liver samples TOH itself and its metabolites accumulate under supplementation (ZHAO *et al.* 2010). The intermediate-chain metabolites, usually detected by GC/MS, are short-lived intermediates and therefore no significant amounts are detected in liquids and tissues (BIRRINGER 2010). Long-chain metabolites, the first metabolic products arising during the catabolism of vitamin E, α-, γ- and δ-13'-OH as well as corresponding 13'-COOH metabolites and subsequent metabolites (11' and 9') have been detected as conjugated or unconjugated structures in cell culture media (JIANG *et al.* 2007; SONTAG & PARKER 2002; YOU *et al.* 2005), human liver cells (BIRRINGER *et al.* 2010; MUSTACICH *et al.* 2010), rat plasma and liver (FREISER & JIANG 2009a; JIANG *et al.* 2007) and rather human liver (SONTAG & PARKER 2002) using GC/MS, HPLC with fluorescence detection, mass spectrometry or rather ion-coupled plasma mass spectrometry, which are suitable for detection of conjugated metabolites (BIRRINGER 2010).

1.1.7.2 Non-antioxidative effects of vitamin E metabolites

Many transcription regulatory properties have been described for α-TOH and for its structure-related isomers and derivatives. Investigation of non-α-TOH compounds showed similar or partly more potent properties compared to α-TOH (GALLI *et al.* 2004a; GALLI *et al.* 2004b; SEN *et al.* 2007). Therefore structurally related compounds are in the focus of current research projects.

Next to their antioxidative capacity (VARGA *et al.* 2008) SCMs are known to mediate antiproliferative effects in PC-3 prostate cancer cells (CONTE *et al.* 2004; GALLI *et al.* 2004b), act antineoplastically and natriuretically (WECHTER *et al.* 1996), reduce the inflammatory response in stimulated rat aortic endothelial cells (RAECs) and mouse microglial cultures (EOC-20) through inhibiting prostaglandin (PG) E_2 and nitric oxide production and release (GRAMMAS *et al.* 2004). Comparable antiinflammatory effects were shown in rats (JIANG & AMES 2003). Jiang and coworkers showed the inhibition of cyclooxygenase activity in murine RAW264.7 macrophages and A549 human epithelial cells (JIANG *et al.* 2000).

Improved analytical procedures allowed the detection of further intermediate metabolites (FREISER & JIANG 2009b; JOHNSON *et al.* 2012). Until now each physiological metabolite arising from hepatic catabolism of vitamin E was found in fluids and/or tissue of humans or animals. Nevertheless the physiological and pathophysiological role and the biological activity of the metabolites with an emphasis on the LCMs are rare because they were not available as pure compounds until recently so that now standards were available. Until know the LCMs have been identified only in human liver cells, human alveolar basal epithelial cell culture medium and rat plasma (BIRRINGER *et al.* 2001; BIRRINGER 2010; FREISER & JIANG 2009a; JIANG *et al.* 2007). Whether the LCMs are present in serum and how they are transported in serum is not

known. Although the chemical synthesis of these LCMs starting from a δ-T3-derivate (trans-13'carboxy-*S*-tocotrienol-*S*'trans-tocotrienoloic acid 2*R*, 3'E, 7'E, 11'E-13'-carboxy-*S*-tocotrienol MAZZINI *et al.* 2009), the so-called *Garcinoic acid* (Figure 10), the main ingredient of an African bitter nut, has been known for some years, first data were published only six years ago. Latest data on the biological activity of the LCM are reviewed in Manuscript II (page 42).

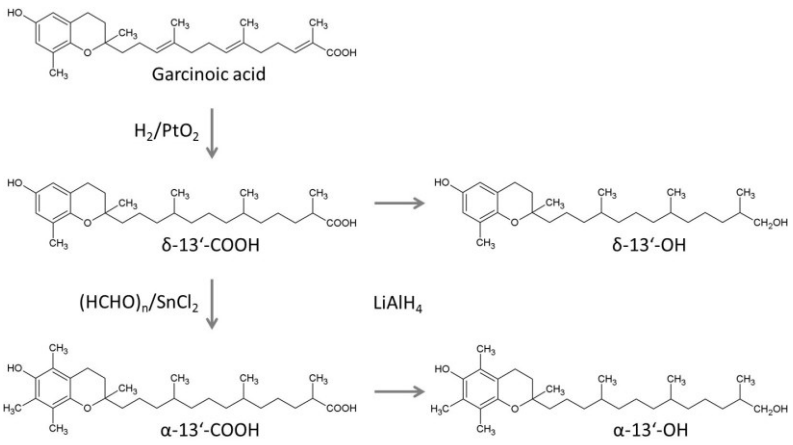

Figure 10: Synthesis of α- and δ-LCM starting from garcinoic acid modification. (BIRRINGER *et al.* 2010)

1.2 Atherosclerosis

Atherosclerosis and its complications such as stroke and myocardial infarction are next to cancer the leading cause of death in Western industrialized societies and are considered as an age-related lifestyle disease. As reviewed by Oliveira and colleagues atherosclerosis starts in childhood or even in fetal development and manifests in the last third or fourth of life-time (OLIVEIRA *et al.* 2010). Since atherosclerotic events have been increasing over the last decades it seems to be a problem of the modern affluent society characterized by an oversupply of food and an unhealthy lifestyle in general. However, there are hints for atherosclerosis being present in ancient times diagnosed as atherosclerotic changes in vessels of mummies (RÜHLI *et al.* 2004; THOMPSON *et al.* 2013). Based on these findings lifestyle and nutrition (DAVID *et al.* 2010) and genetic predispositions are main risk factors of atherosclerosis next to age (OLIVEIRA *et al.* 2010).

1.2.1 Atherogenesis

Atherosclerosis is a progressive inflammatory disease of middle and large-sized arteries characterized by extensive morphological changes of the vascular wall. The pathogenesis of atherosclerosis is initiated by endogenous and exogenous noxes accompanied by endothelial dysfunctions which lead to intimal thickening caused by T-cell and monocyte recruitment as well as their migration into the subintimal space. In brief subsequent progressive events in atherosclerosis are macrophage differentiation, immigration of SMCs, lipid accumulation in macrophages and proliferation of lipid-loaded macrophages, deposition of cholesterol and fibrotic material, foam cell formation, fatty streak formation, elasticity loss and vascular

lumen reduction with parallel widening of the arterial diameter (WALLERT *et al.* 2014). Taken together these events progress over decades resulting in the formation of a necrotic lipid core with a fibrotic cap which is destabilized by proteases released by macrophages. Finally plaque rupture followed by thrombus formation may take place. Subsequently two possible pathways are conceivable: the thrombus (i) flows downstream with the blood to small volume arteries and causes vascular occlusions, or (ii) it remains at the plaque site where it may also occlude the artery. Incorporation of the thrombus at the plaque site leads to the formation of the so-called complicated plaque (WALLERT *et al.* 2014). Risk factors and the initiation and propagation of atherosclerosis are described in more detail in Manuscript II (see page 42).

1.2.2 Macrophages

In inflammatory and lipid metabolism associated diseases such as atherosclerosis macrophages, the phagocytic cells of the immune system, play a pivotal role. In brief, they control atheroma development by releasing cytokines, ROS, and proteases resulting in weakening and destabilization of the fibrous cap (LIBBY 2002). Additionally macrophages accumulate large amounts of cholesterol esters (GOLDSTEIN *et al.* 1979a) and triglycerides and mobilize cholesterol for reverse cholesterol transport (GHOSH *et al.* 2010). Since excessive lipid accumulation leads to foam cell formation, macrophages contribute actively to atherosclerosis. In parallel macrophages release signaling molecules such as chemotactic mediators resulting in recruitment of other immune cells as well as SMCs (LIBBY 2002).

Functional activation of macrophages as a result of the immune response to microbial components such as lipopolysaccharides (LPS) modifies their repertoire of pattern-recognition receptors and this is called macrophages polarization (BISWAS & MANTOVANI 2010). For years the concept of polarization has distinguished macrophages into two extreme activation states named classical (M1) or alternative (M2a, b, c) activation with distinct characteristics which can be switched upon appropriate stimuli (BISWAS & MANTOVANI 2010; CHINETTI-GBAGUIDI & STAELS 2011; HOEKSEMA *et al.* 2012). Classically polarized macrophages (M1) are distinguished from M2 by the induction of inflammatory response genes (XUE *et al.* 2014) and transcription factors such as signal transducers and activators of transcription (STAT) family type 1, nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) p65 subunit, peroxisome proliferator-activated receptor (PPAR) γ and liver X receptor (LXR) α (HOEKSEMA *et al.* 2012) as well as induction phagocytic activity and a distinct cytokine and chemokine profile (BISWAS & MANTOVANI 2010). Macrophage subtypes can switch between polarization status M1 and the different M2 states depending on the exogenous stimuli (CHINETTI-GBAGUIDI *et al.* 2011; HOEKSEMA *et al.* 2012). To what extent monocyte subtypes influence macrophage polarization is questionable. In comparison classically activated macrophages can be characterized as proinflammatory whereas alternatively activated macrophages mainly mediate antiinflammatory events and contribute to plaque stability, tissue remodeling and repair (CHINETTI-GBAGUIDI & STAELS 2011; FEIG *et al.* 2011). Subsequently the main functional classification of macrophages into classically activated

macrophages, wound-healing and regulatory macrophages was extended by intermediate forms with shared characteristics such as tumor-associated macrophages (MOSSER & EDWARDS 2008). In continuation this spectrum model was promoted by Xue *et al.* who described at least nine distinct macrophage activation programs under inflammatory conditions which supports the notion of macrophages as a very heterogeneous cell population (CHINETTI-GBAGUIDI & STAELS 2011; XUE *et al.* 2014). Macrophages differing in activation status show a stimulation-related gene expression signature represented in differences in cell surface markers and patterns of involved transcription factors (XUE *et al.* 2014).

The orchestration of macrophage function plays a pivotal role in different physiological but in particular pathological conditions such as the development and progression of atherosclerotic plaques (BISWAS & MANTOVANI 2010; CHINETTI-GBAGUIDI & STAELS 2011). The induction of M2 associated gene expression goes along with atherosclerotic plaque development whereas M1-related markers are increased in very early lesions (CHINETTI-GBAGUIDI & STAELS 2011). A macrophage phenotype distinct from M1 and M2 activation status namely Mox caused by oxidized phospholipids exists in atherosclerotic lesion of mice and man represents about 30% of all macrophages (CHINETTI-GBAGUIDI & STAELS 2011). M1 macrophages are present in human carotid atherosclerotic lesions, whereas M2 macrophages prevail in the shoulder region as well as in the periphery of the plaque (CHINETTI-GBAGUIDI *et al.* 2011; CHINETTI-GBAGUIDI & STAELS 2011). Macrophages may contain different kind of cytosolic lipid droplets depending on their activation: either many but small (M2) or fewer but bigger (M1) lipid droplets (CHINETTI-GBAGUIDI *et al.* 2011).

1.2.3 Macrophage foam cell formation

Dysbalanced lipid homeostasis as a combination of excessive lipid uptake and decreased export of lipids results in the formation of foam cells which is a hallmark of atherosclerosis (Figure 11). Macrophage lipid uptake mechanisms involved several surface-associated receptors, such as SR and lipoprotein receptors, both mediating lipid uptake and phagocytosis. Scavenger receptors, such as the cluster of differentiation (CD) 68 and the lectin-like oxidized low density lipoprotein receptor 1, contribute to the uptake of modified LDL via phagocytosis (SCHRIJVERS *et al.* 2007). The LDLR mainly binds native LDL (nLDL). Since the incorporated nLDL blocks its own further uptake via the LDLR, nLDL-derived cholesterol does not contribute significantly to foam cell formation of macrophages. Additionally, LDLR mediates the endocytosis of so-called minimally oxidized LDL (mmLDL) formed by 12/15-lipoxygenases (LOX) (TSIMIKAS & MILLER 2011). However, the modification of LDL by myeloperoxidase (MPO) transforms it into an SR-A ligand (WANG *et al.* 2007). Low density lipoprotein receptor-related protein (LRP) 1 initiates cholesteryl ester (CE) exchange between LDL and the cell resulting in 12/15LOX-mediated oxygenation of CE and contributes further to the efflux of oxidized cholesterol back to the LDL particle (TSIMIKAS & MILLER 2011). The contribution of these different modified LDL particles and their receptors to foam cell formation is probably less significant than that of oxidized LDL (oxLDL) and their receptor CD36. Within the scavenger receptor family, CD36 plays a pivotal role in foam cell formation

by binding and initiation of uptake of oxLDL efficiently to a large extent (COLLOT-TEIXEIRA *et al.* 2007). The oxidation of LDL occurs *in vivo* primarily through ferrous ions (Fe^{2+}). Contrary to the uptake of nLDL the uptake of oxLDL is not subjected to negative feedback regulation which results in uncontrolled oxLDL uptake by macrophages. Subsequently starting from the Golgi apparatus lipid droplets containing CE, phospholipids, free cholesterol (FC) and

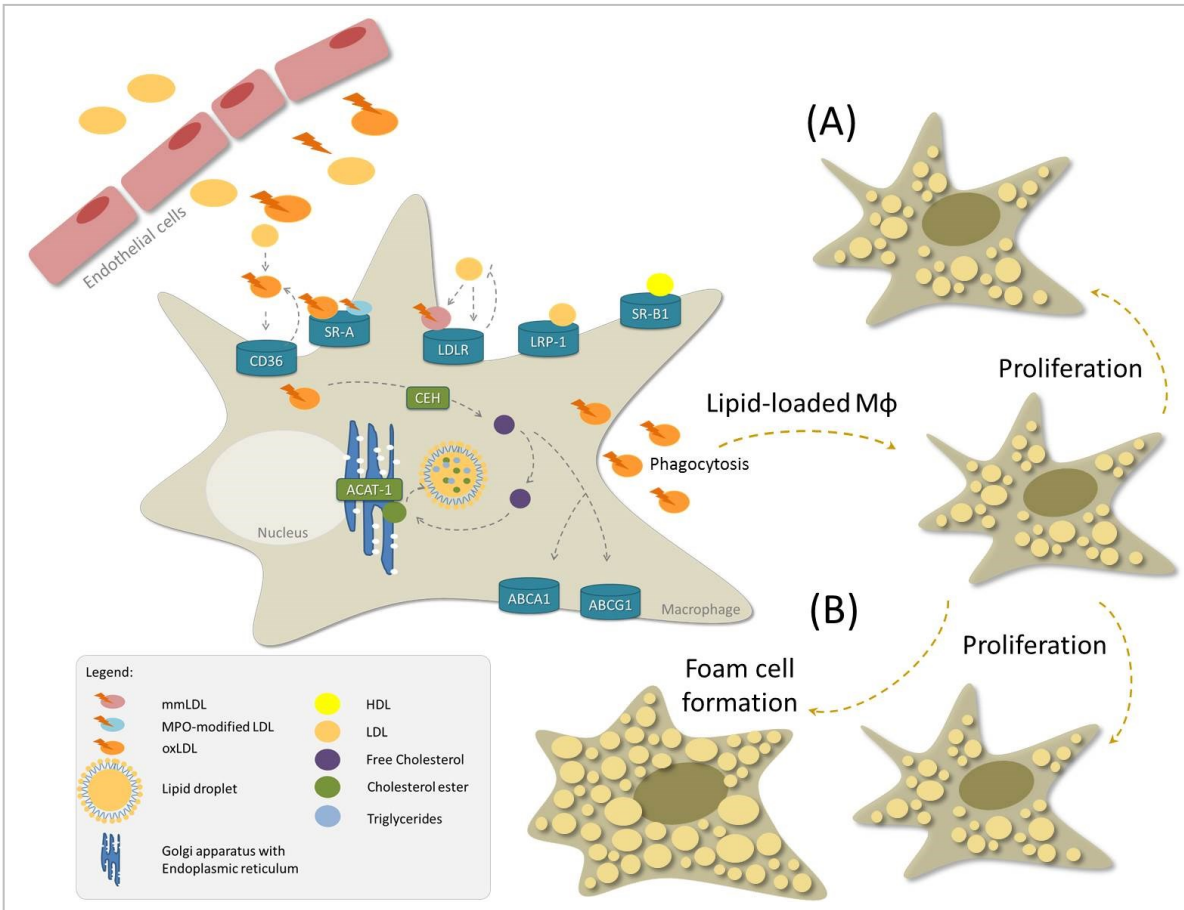

Figure 11: Foam cell formation. Lipid accumulation in macrophages is caused by a dysbalance between import and export of cholesterol. Enhanced cholesterol import, particularly LDL, can be caused by endothelial dysfunction which causes deposition of cholesterol-rich LDL within the arterial walls. Following these LDL particles are modified, predominantly oxidation by enzymes or radicals occurs. This plays a key role in atherogenesis. The uptake of modified LDL via different types of scavenger receptors is not feedback-regulated in contrast to the uptake of native LDL uptake via LDLR. Important receptors regulating the import of modified LDL are CD36 and SR-A which mainly bind oxLDL but also MPO-modified LDL. Additionally oxLDL can be taken up by phagocytosis. Since the lipid export mechanisms fail to balance the enhanced cholesterol uptake, overwhelming lipid droplet formation is observed. This results in lipid loaded macrophages which (A) proliferate and (B) form foam cells, a hallmark of atherosclerosis. (GHOSH *et al.* 2010; ROBBINS *et al.* 2013; TSIMIKAS & MILLER 2011)

triglycerides are formed (TSIMIKAS & MILLER 2011). Cholesteryl ester as the main component of LDL and oxLDL are hydrolyzed in late endosomes/lysosomes by the cholesteryl ester hydrolase (CEH) to FC (GHOSH *et al.* 2010), this is called cholesterol ester cycle (BROWN *et al.* 1980). Free cholesterol is again transported back to the endoplasmic reticulum where it is re-esterified by the acetyl CoA acetyl transferase (ACAT) 1 and stored in lipid droplets. Since only FC is cytotoxic for the cells the CE are less harmful. However only FC can be transported by ABCA1 and G1 and therefore esterification inhibits FC export (BUHMAN *et al.* 2000;

PRAMFALK *et al.* 2012). Based on their involvement in cholesterol export the HDL receptors, ABCA1 and probably SR-B1, are classified as antiatherogenic (FITZGERALD *et al.* 2010).

1.2.4 Inflammation

Inflammation is an important part of many immune response-related diseases, such as rheumatoid arthritis, psoriasis and systemic lupus erythematosus, and is further assigned as a key risk factor for atherosclerosis, especially if chronic inflammatory diseases pre-exist (HANSSON & HERMANSSON 2011). Fundamental processes taking place during inflammation in macrophages are cytoskeletal rearrangement, macropinocytosis, increased ROS regeneration, reduced phagocytic capacity towards apoptotic cells, expression of proinflammatory genes, and the release of chemokines and cytokines, involvement of

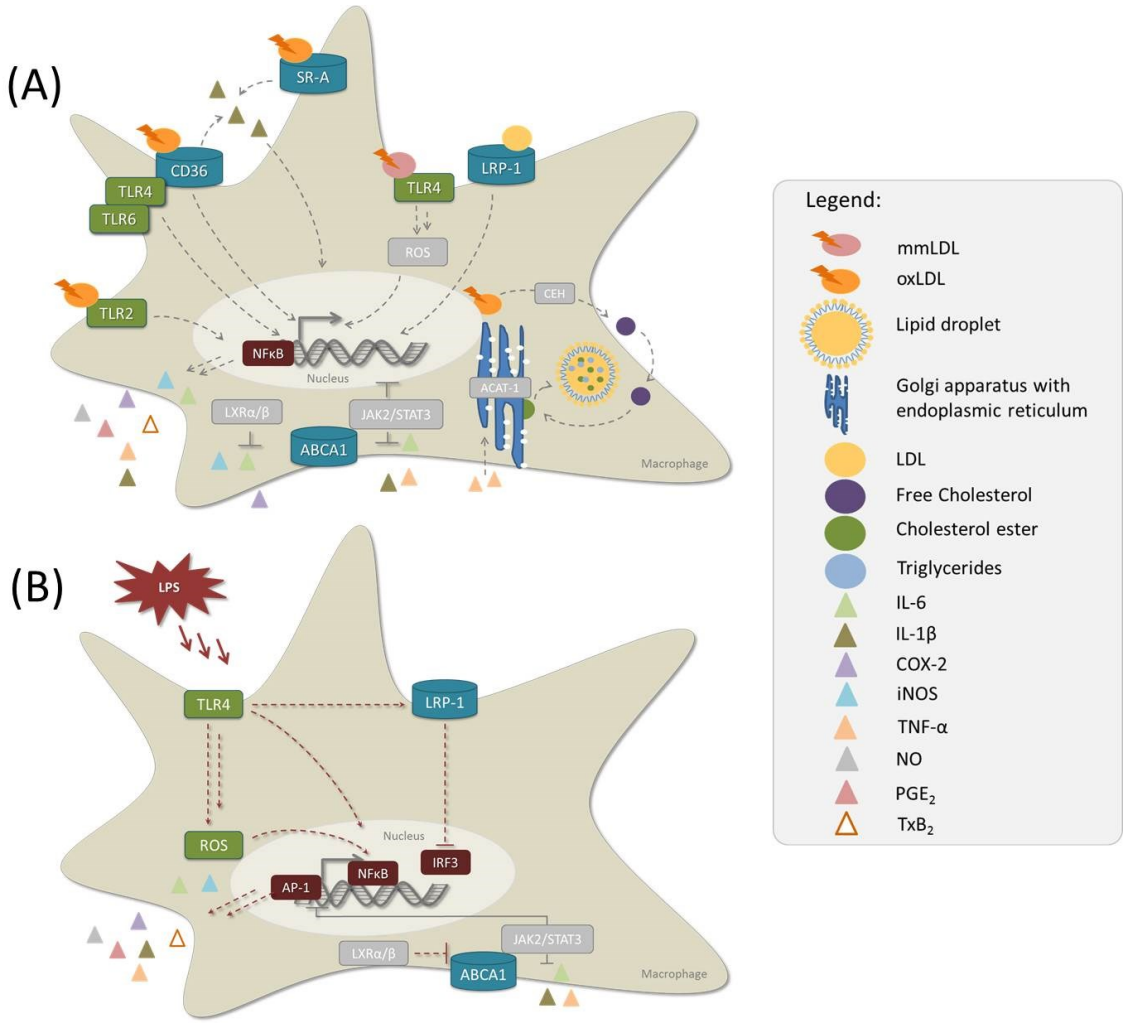

Figure 12: (A) Lipoprotein and (B) lipopolysaccharide mediated inflammatory pathways. Inflammation caused by both modified LDL and LPS induces pro-inflammatory pathways contribute to the propagation of inflammatory diseases such as atherosclerosis. Important receptors are scavenger receptors and Toll-like receptors which further enhance the inflammatory response by regulating signal transduction as well as production and secretion of cytokines and chemokines. (FITZGERALD *et al.* 2010; HANSSON & HERMANSSON 2011; TSIMIKAS & MILLER 2011)

transcription factors, such as NFκB, activator protein (AP) 1, interferon regulatory factor (IRF) 3, LXR-α/β and PPARγ (TSIMIKAS & MILLER 2011) (Figure 12). Inflammatory activation of macrophages by both bacterial components and excessive lipid uptake is known. Modified lipoproteins are initiators of inflammation and cause directly and independently lipid accumulation. As described above native LDL is taken up by the LDLR. Additionally the LDLR binds mmLDL which subsequently modulate NFκB signaling, resulting in nitric oxide synthase (NOS) and NADPH oxidase (NOX) regulation and proinflammatory cytokine release such as interleukin (IL)-1β, IL-6 and RANTES (regulated on activation, normal T cell expressed and secreted) (TSIMIKAS & MILLER 2011). Low density lipoprotein is further a ligand of LRP-1, which directly regulates inflammatory signaling, and cytokine secretion (MAY 2013). Furthermore, mmLDL can be recognized by Toll-like receptor (TLR) 4 but with lower priority compared to LPS, the major ligand for TLR4 (TSIMIKAS & MILLER 2011). In general TLR2, TLR4 and TLR6 are key receptors regulating the inflammatory response of macrophages. Next to mmLDL MPO-modified LDL can be phagocytized by macrophages via scavenger receptors CD36 and SR-A (TSIMIKAS & MILLER 2011). Oxidized LDL, known to contribute to the formation of foam cells, plays also an important role in the inflammatory response. Initial uptake of oxLDL by CD36 mediates dimerization of CD36 with TLR4 and TLR6 thus activating tyrosine kinase Lyn. This ultimately leads to interference with NFκB signaling by myeloid differentiation primary response gene (MyD) 88 and TIR-domain-containing adapter-inducing interferon-β (TRIF). This finally causes the release of ROS, chemokines and cytokines (TSIMIKAS & MILLER 2011). The most important receptor for reverse cholesterol transport via HDL, SR-B1, can suppress LPS-induced cytokine secretion in macrophages through inhibition of TLR4-mediated NFκB activation (GUO *et al.* 2009).

Regulation of lipid homeostasis requires balanced import via CD36, SR-A, SR-B1 and export mechanisms via ABCA1/G1. Increased capacity of receptors modulating cholesterol efflux through the reverse cholesterol transport pathway such as ABCA1 and ABCG1 decrease cholesterol levels within macrophages. Therefore ABCA1 and ABCG1 were assumed as anti-inflammatory receptors playing a pivotal role in the inflammatory response (FITZGERALD *et al.* 2010). Whether the antiinflammatory properties are a consequence of their lipid transport activity or direct interaction with STAT3 and Janus kinase (JAK) 2 is not known. Possibly the ABCA1 with apoA-1 interaction suppresses secretion of inflammatory cytokines in response to LPS stimulation or acts directly as an antiinflammatory receptor by stimulating the JAK2/STAT3 pathway, known as a repressor of inflammatory cytokine production (TANG *et al.* 2009). Additionally, further transcription factors, namely PPARγ and LXRα/β, were described to repress transcriptional activation of inflammatory response genes such as inducible NOS (iNOS) (JOSEPH *et al.* 2003; PASCUAL *et al.* 2005).

2 Aim of the Study

Apart from essential macronutrients balanced vitamin and mineral supply is a key issue in the current discussion about a healthy diet. In the case of vitamin and mineral intake many people believe that the more is the better (DWYER *et al.* 2014). Vitamin and mineral supplementation as well as enrichment in food increased over last decades (DWYER *et al.* 2014). Vitamin E, especially α-tocopherol (α-TOH), is the most important lipid antioxidant which is widely used to prevent age-associated diseases, such as cardiovascular diseases (CVD), including atherosclerosis (BIRRINGER 2010). Despite increasing knowledge about human vitamin E metabolism little is known to justify its widespread use. Since meta-analyses revealed even harmful effects of high vitamin E doses and a lack of protection against cardiovascular events, a profound understanding of vitamin E metabolism is mandatory (MILLER 2005; WALLERT *et al.* 2013 // 2014).

The term vitamin E describes a group of isomers of which α-TOH is assigned as the most important vitamer. In regard to preventing cardiovascular complications studies have revealed controversial results and failed to demonstrate clear invers correlations or positive effects of vitamin E supplementation (WALLERT *et al.* 2014). One possible explanation for the different outcomes of human intervention studies could be that individual differences in vitamin E metabolism exist. Therefore the importance of metabolic differences in vitamin E metabolism needs to be clarified further.

A complete knowledge about the metabolism of vitamin E with respect to the physiological properties of the metabolites α-13‘-OH and α-13‘-COOH formed from α-TOH is a prerequisite for future evaluations about antiatherogenic properties of vitamin E. Therefore the aim of this thesis was to contribute to a better understanding of the molecular modes of actions of the long-chain metabolites of α-TOH (α-LCM). In particular this thesis will focus on the detection of the α-LCMs in serum and on investigations of their regulatory features.

Since several vitamin E metabolites have been detected in human serum and tissues except the α-LCMs α-13‘-OH and α-13‘-COOH (BIRRINGER 2010), successful detection of these metabolites will provide first evidence for a physiological relevance and will be the basis for further investigations. Thereby it is necessary to develop and optimize suitable analytical protocols including sample preparation. Analytical techniques such as HPLC, GC/MS and LC/MS will be tested for the detection of the LCMs.

Since macrophages are of particular importance in atherogenesis the impact of the α-LCMs on human and murine macrophages will be investigated using different types of cell models. Macrophage foam cell formation is a key event in atherosclerosis which was reported to be influenced by α-TOH. Thus the effects of the LCMs on macrophage foam cell formation will be investigated.

Next to foam cell formation inflammation plays a pivotal role in atherogenesis. Since murine macrophages are an established model for investigating the inflammatory response LPS-activated murine RAW264.7 cells will be used for investigating the antiinflammatory

properties of the LCMs.

Overall this thesis intends to elucidate the impact of the α-LCMs on atherogenic processes and macrophage activation, particularly in comparison to the parent compound α-TOH. Distinct regulatory capacity of the metabolites in combination with individual differences in α-TOH metabolism and transport may provide adequate explanations for contradictory results obtained from studies of α-TOH supplementation. This information will be a vital contribution for an accurate assessment of the contribution and molecular modes of action of vitamin E for the prevention of cardiovascular complications.

3 Manuscript overview

Manuscript I:

Long-chain metabolites of α-tocopherol occur in human serum and inhibit macrophage foam cell formation *in vitro*.

Wallert M, Mosig S, Rennert K, Funke H, Ristow M, Pellegrino RM, Cruciani G, Galli F, Lorkowski S, Birringer M

Free Radical Biology and Medicine (2014); 68C:43-51

DOI: 10.1016/j.freeradbiomed.2013.11.009

Date of acceptance: 13th November 2013

Personal contribution to the publication:

Maria Wallert:	Performance of RT-qPCR, flow cytometry and lipoprotein as well as microbead uptake experiments, writing of the manuscript, analysis of data; optimization of uptake experiments Total contribution: 80%
Sandy Mosig:	Training of methods and supervision of flow cytometry studies, statistical calculations of flow cytometry experiments
Knut Rennert:	Training of methods and supervision of flow cytometry studies, preparation of lipoproteins, set-up of uptake experiments
Harald Funke:	Writing and proofreading of manuscript, taking blood samples
Michael Ristow:	Writing and proofreading of manuscript
Roberto Maria Pellegrino:	Performance of LC/MS-QTOF experiments, analysis of data, establishment of protocol for metabolite analysis
Gabriele Cruciani:	Proofreading of manuscript, analysis of LC/MS-QTOF data
Francesco Galli:	Design of serum analysis experiments, training of methods and supervision of analytical studies, analysis of LC/MS-QTOF data
Stefan Lorkowski:	Designed the study, analyzed the data, evaluated the results, proof-read the manuscript

Marc Birringer:	Designed the study, analyzed the data, evaluated the results, proof-read the manuscript

Key messages of Manuscript I:

We detected α-13'-COOH for the first time in human serum by using LC/MS-QTOF. Thus these findings provided evidence for systemic bioavailability of this hepatic long-chain metabolite of α-TOH. Our study reports the relevance of long-chain metabolites, α-13'-COOH and its precursor α-13'-OH, on foam cell formation, a hallmark of atherosclerosis. The major scavenger receptor for the uptake of oxLDL, CD36, is increased by α-13'-COOH and α-13'-OH in contrast to α-TOH, although the uptake of oxLDL and lipid accumulation in presence of α-13'-COOH and α-13'-OH is decreased. This discrepancy may be explained by decreased phagocytic activity observed in this study. In summary we assume that the long-chain metabolites of α-TOH represent a new class of regulatory metabolites, which act more potently in the regulation of molecular pathways partly distinct from their precursor α-TOH.

Manuscript II:

Regulatory metabolites of vitamin E and their putative relevance for atherogenesis.

Wallert M, Schmölz L, Galli F, Birringer M, Lorkowski S

Redox Biology (2014); 2:495-503

DOI: 10.1016/j.redox.2014.02.002

Date of acceptance: 11th February 2014

Personal contribution to the publication:

Maria Wallert:	Writing of the manuscript, collection and evaluation of data, concept development Total contribution: 40%
Lisa Schmölz:	Writing of manuscript, collection and evaluation of data, concept development
Francesco Galli:	Proofreading of the manuscript
Marc Birringer:	Writing and proofreading of the manuscript, concept development
Stefan Lorkowski:	Writing and proofreading of the manuscript, concept development

Key messages of Manuscript II:

Vitamin E, a group of different isomers of with α-TOH is assigned as the most active vitamer, is likely the most important antioxidant in human diet. Beside antioxidative capacity α-TOH exhibits antiinflammatory activity *in vitro*. Despite promising antiatherogenic features *in vitro*, vitamin E failed to be atheroprotective in clinical trials in humans for several reasons. We highlighted a role of the physiological long-chain metabolite α-13'-COOH of α-TOH as a proposed new class of regulatory metabolites which complicate the mode of action of α-TOH. Since the formation of α-13'-COOH is probably interindividually different this could be a reason for the discrepancies of the results from *in vitro* and *in vivo* studies.

Manuscript III:

The α-tocopherol long-chain metabolite α-13'-COOH affects the inflammatory burst of lipopolysaccharide-activated murine RAW264.7 macrophages.

Wallert M, Schmölz L, Glei M, Krauth V, Werz O, Birringer M, Lorkowski S

European Journal of Nutrition

Date of submission: 27th June 2014

Personal contribution to the publication:

Maria Wallert:	Design and performance of RT-qPCR experiments, Cox2 and iNos Western blots and Griess assays, writing of manuscript, analysis of data, concept development Total contribution: 60%
Lisa Schmölz:	Performance of NFκB Western blots, analysis of data and evaluation of results
Michael Glei:	Proofreading of manuscript, analysis of data, evaluation of results
Verena Krauth:	Performance of PGE_2 ELISAs, analysis of data
Oliver Werz:	Proofreading of manuscript, analysis of data, evaluation of results
Marc Birringer:	Proofreading of manuscript, concept development, analysis of data, evaluated the results
Stefan Lorkowski:	Design of study, proofreading of manuscript, concept development, analysis of data and evaluation of results

Key messages of Manuscript III:

Inflammatory response as key initiator and driving force for many age associated diseases, such as atherosclerosis, is suppressed by α-TOH and its long-chain metabolite α-13'-COOH in murine macrophages. A screening of pro- and antiinflammatory markers using RT-qPCR and Western blot analyses showed a significant decrease of Il10, Cox2 and iNos by α-TOH and more pronouncedly by α-13'-COOH. Likely due to reduction of Cox2 and iNos expression production of signal molecules PGE_2 and nitric oxide was significantly reduced. Observed blocking of inflammatory pathways by α-13'-COOH may be independent from NFκB. Our

study sheds new light on α-TOH mode of action in inflammatory pathways in macrophages, mediated possibly by its physiological LCM α-13'-COOH which appears to regulate distinct pathways.

Manuscript IV:

First analysis of α-13'-COOH (13'(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid) a long-chain metabolite of vitamin E in human serum.

Wallert M, Ciffolilli S, Galli F, Birringer M, Lorkowski S

Suggested Journal: Journal of Lipid Research - Method Section

Personal contribution to the publication:

Maria Wallert:	Performance of sample preparation, HPLC, GC/MS measurements, writing of manuscript, analysis of data, concept development, optimization of serum extraction protocol Total contribution: 60%
Silvia Ciffolilli:	Performance of analytical studies (HPLC), preparation of samples and measurement of standards, establishment of basic protocol
Francesco Galli:	Training of methods and supervision of analytical studies, proofreading of manuscript, evaluation of results
Marc Birringer:	Proofreading of manuscript, concept development, analysis of data
Stefan Lorkowski:	Proofreading of manuscript, concept development, analysis of data and evaluation of results

Key messages of Manuscript VI:

Metabolism of vitamin E has not been fully understood yet. In the liver, α-tocopherol is metabolized by CYP4F2/CYP3A4-dependent ω-hydroxylation followed by α-oxidation in peroxisomes, which results in the formation of intermediate long-chain metabolite carboxychromanol (α-13'-COOH). Due to the optimization of the preparation protocols regarding enzymatic processing, solvent for organic extraction and the used amount of serum, we detected α-13'-COOH in human serum by LC/MS-QTOF at baseline and after one week supplementation of *RRR*-α-TOH for the first time. This represents an important analytical advancement in the study of vitamin E metabolism in humans and provides evidence for the physiological relevance of this long-chain metabolite.

3.1 Manuscript I

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Original Contribution

Long-chain metabolites of α-tocopherol occur in human serum and inhibit macrophage foam cell formation in vitro

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Free radicals

ABSTRACT

Despite intensive research the physiological role and molecular mechanisms of action of the lipophilic antioxidant α-tocopherol (α-TOH) are still poorly understood. Hepatic α-TOH catabolism results in intermediate formation of the long-chain metabolites (α-LCMs) α-13′-hydroxy- and α-13′-carboxychromanol (α-13′-OH and α-13′-COOH). We propose that α-LCMs have biological functions that need further exploration. Here we report that α-13′-COOH, as detected by LC/MS Q-TOF, occurs in human serum, providing evidence for its systemic bioavailability. Using semisynthetically derived α-LCMs we performed flow cytometric analyses and found that α-LCMs decrease oxidized LDL (oxLDL) uptake (α-13′-OH, 24 ± 6%, α-13′-COOH, 20 ± 5% vs control) and oxLDL-induced lipid accumulation in human macrophages in vitro (α-13′-OH, 26 ± 4%, α-13′-COOH, 21 ± 9% vs oxLDL), probably owing to α-LCM-mediated reduction in phagocytosis of oxLDL (α-13′-OH, 16 ± 6%, α-13′-COOH, 41 ± 3% vs oxLDL). At the same time, α-LCMs induced expression of CD36, the major scavenger receptor for oxLDL, in human macrophages by about 4.5-fold. Blocking experiments provided evidence that α-LCMs influence oxLDL uptake independent of CD36. A key finding of our study is that bioactivity of the α-LCMs occurs at lower concentrations and with mechanisms distinct from those of their metabolic precursor α-TOH. Our findings shed new light on the mechanistic aspects of α-TOH function in macrophages, which seem to be complicated by circulating α-LCMs. We speculate that α-LCMs represent a new class of regulatory metabolites. Further studies are required to elucidate their physiological role and contribution to cardiovascular disease.

Atherosclerosis is a progressive inflammatory disease characterized by extensive morphological changes in the vascular wall, such as intimal thickening, cholesterol deposition, elasticity loss, and vascular lumen reduction [1]. More than 50% of all mortalities in Western societies are caused by atherosclerosis and its complications [2].

Macrophages are the phagocytic cells of the immune system, which play a pivotal role in inflammatory disease processes such as atherosclerosis. In addition to contributing to wound healing and antigen presentation [3], macrophages engage actively in lipid metabolism by storing large amounts of cholesterol esters and triglycerides and by mobilizing cholesterol for reverse cholesterol transport [4,5]. Excessive lipid accumulation leads to macrophage foam cell formation and release of chemotactic mediators [6].

CD36 plays an important role in atherogenesis because uptake of oxidized LDL (oxLDL)[4] via this scavenger receptor is not subjected to negative feedback and contributes significantly to macrophage foam cell formation [7]. Scavenger receptors such as

Abbreviations: α-LCM, α-tocopherol long-chain metabolite; α-TOH, α-tocopherol; α-13′-OH, α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyltridecanol; α-13′-COOH, α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyltridecanoic acid; nLDL, native low-density lipoprotein; oxLDL, oxidized low-density lipoprotein

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CD68 and LOX-1 contribute to the uptake of modified LDL via phagocytosis [8].

The term vitamin E describes a mixture of eight isomers that differ by methylation patterns of the hydroxychromanol ring and saturation of the side chain (α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol) and of which α-tocopherol (α-TOH) is the most active vitamer [9]. Vitamin E is the most important lipid antioxidant that is widely used to prevent age-associated diseases [10]. In addition to its anti-inflammatory properties, vitamin E modulates gene expression, e.g., expression of proteins involved in uptake, transport, and degradation of tocopherols, such as α-TTP and CYP3A4 [11] and/or CYP4F2 [12]; lipoprotein uptake, such as SR-A and SR-B [13]; inflammation; and regulation of signal transduction, e.g., via PPARγ [14,15].

In the liver, α-TOH is metabolized by side-chain truncation initiated by CYP3A4-dependent ω-hydroxylation, which results in the formation of the alcohol derivate α-13′-OH (13′-(6-hydroxy-2, 5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyltridecanol) [16]. Subsequent α-oxidation in peroxisomes forms the acid derivative α-13′-COOH (13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2, 6,10-trimethyltridecanoic acid). The main short-chain α-TOH metabolite, the end-product of α-TOH catabolism, is α-carboxyethylhydroxychroman (α-CEHC), which is excreted via urine (Supplementary Fig. S1) [17,18].

The long-chain metabolites (α-LCMs) of α-TOH, α-13′-OH and α-13′-COOH, which have been identified so far only in human liver cells and feces [16], may display biological properties that are distinct from those of α-TOH. However, detailed information about the physiological functions of α-LCMs and their contribution to atherogenesis are scarce because they were not available as pure compounds until recently.

Here we report for the first time on the detection of α-13′-COOH in human plasma and the modulation of macrophage foam cell formation by α-13′-OH and α-13′-COOH. The results presented here provide the first evidence for a new molecular mode of action of α-TOH via its α-LCMs α-13′-OH and α-13′-COOH.

Materials and methods

Cell culture

THP-1 monocytes and macrophages

THP-1 monocytes (ATCC, Manassas, VA, USA) were cultivated in RPMI 1640 (PAA Laboratories, Coelbe, Germany) supplemented with 10% fetal bovine serum and 0.1 mg/ml penicillin/streptomycin/L-glutamine mixture (PAA Laboratories) as described [19]. Cells were cultured at 37 °C in a humidified 5% CO_2/95% air atmosphere. Differentiation into macrophages was initiated by adding 100 ng/ml phorbol 12-myristate 13-acetate (Fisher Scientific, Schwerte, Germany) and 50 μmol/L β-mercaptoethanol (Roth, Karlsruhe, Germany) to supplemented RPMI 1640 according to standard protocols [20]. After 96 h fully matured macrophages were incubated with compounds as indicated in the figures and harvested for further processing as described below.

Human monocyte-derived macrophages

Human monocytes were isolated from the blood of healthy volunteers using RosetteSep Monocyte Enrichment Cocktail (Cell Systems, St. Katharinen, Germany) according to the manufacturer's protocol. After cell purity was confirmed by flow cytometry with antibodies directed against CD14 (monocytes), CD3 (T cells), CD235a (erythrocytes), CD19 (B cells), CD41a (platelets), and CD56 (natural killer cells), cell viability was controlled. Isolated monocytes were differentiated into macrophages for 6 days at 37 °C in a humidified 5% CO_2/95% air atmosphere in X-VIVO 15 (Cambrex, Walkersville, MD, USA) supplemented with 20% human serum derived from the respective donor or with pooled human serum (PAA Laboratories), 0.2% normocin (Biomol, Hamburg, Germany), 0.1 mg/ml streptomycin/gentamycin mixture (Lonza, Cologne, Germany), and 10 ng/ml GM-CSF (Cell Signaling Technology, Danvers, MA, USA). For experiments macrophages were incubated with compounds for 24 h in supplemented but serum-free X-VIVO 15 as indicated in the figures.

Human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from human blood by Ficoll density gradient centrifugation as described [21]. Blood was mixed 1:1 with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 2 mmol/L EDTA. Fifteen milliliters of Ficoll (Biomol) was overlaid with 30 ml of blood–PBS mixture and centrifuged at 800 *g* for 20 min. PBMCs were collected and washed twice with cold PBS supplemented with 0.1% BSA and 2 mmol/L EDTA. The PBMCs were seeded in supplemented serum-free X-VIVO 15. After 2 h incubation at 37 °C in a humidified 5% CO_2/95% air atmosphere the cells were washed. Adherent macrophages were differentiated for 6 days in X-VIVO 15 supplemented with 20% human serum, 0.2% normocin, 0.1 mg/ml streptomycin/gentamycin mixture, and 10 ng/ml GM-CSF. For experiments cells were cultured in supplemented serum-free X-VIVO 15 in the presence or absence of compounds as indicated in the figures.

RNA isolation and cDNA synthesis

Total RNA was isolated from cell lysates using a Qiagen RNeasy Mini kit (Hilden, Germany) including on-column DNase I digestion (Qiagen) as described [22]. cDNA synthesis was performed using a Revert Aid first-strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany), 5 μg of total RNA, and 500 ng/μl oligo(dT) primers as described [23].

Quantitative real-time RT-PCR (RT-qPCR)

RT-qPCR was performed on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using a QuantiTect SYBR Green PCR kit (Qiagen) as described [23]. Primers (CD36, RPL37A, Supplementary Table S1) were from Invitrogen (Karlsruhe, Germany). PCR runs included a 15-min preincubation at 95 °C, followed by a 40-cycle two-step PCR consisting of a denaturing phase at 94 °C for 15 s and a combined annealing and extension phase at 60 °C for 30 s. PCR results were analyzed using the LightCycler software 1.5.0.39.

LDL preparation and labeling

LDL was isolated from the serum of healthy and normolipidemic volunteers by sequential ultracentrifugation [24] and stored in 0.02% NaN_3, Na_2EDTA (0.24 mmol/L) at 4 °C under a N_2 atmosphere. Oxidation of LDL was achieved by a 6-h incubation of native LDL with 10 μmol/L $CuSO_4$ at 37 °C. Oxidized LDL was desalted by PD-10 desalting columns (GE Healthcare, Uppsala, Sweden) and eluted in PBS. Native LDL (nLDL) and oxLDL were labeled using an AlexaFluor488 protein labeling kit (Molecular Probes, Leiden, Netherlands) according to the manufacturer's protocol. All lipoprotein preparations were endotoxin low (< 0.02 EU/ml) as confirmed by a QCL-1000 Chromogenic LAL kit (Cambrex). LDL protein content was measured using a modified Lowry protein assay (Bio-Rad, Munich, Germany).

Flow cytometry

Measurement of CD36 protein expression

THP-1 cells or human primary macrophages were cultured and matured. After 24 h incubation with α-TOH, α-13′-OH, or α-13′-COOH as indicated in the figures adherent cells were washed with PBS and detached with lidocaine–EDTA solution containing 4 mg/ml lidocaine hydrochloride and 5 mmol/L EDTA (Sigma–Aldrich, Seelze, Germany). THP-1 cells were analyzed on a FACSCalibur (BD Biosciences); pure human macrophages were measured on a FACSCanto II (BD Biosciences). Anti-human FITC-labeled CD36 antibody (Beckman Coulter, Krefeld, Germany) and anti-human FITC-labeled mIgG antibody (Miltenyi Biotech, Bergisch Gladbach, Germany) were added to a final concentration of 5 μg/ml for 30 min at 37 °C and 5% CO_2. Data were analyzed with FlowJo software 9 (TreeStar, Ashland, OR, USA).

The same protocol was used to investigate the influence of α-TOH and its long-chain metabolites on CD36 expression in the presence of lipoproteins, 50 mg/L nLDL or oxLDL, for 24 h.

Analysis of lipoprotein uptake

THP-1 cells or human monocyte-derived macrophages were cultured and differentiated. After 24 h incubation with 10 μmol/L α-13′-OH and 5 μmol/L α-13′-COOH, 50 mg/L oxLDL (10 mg/L AlexaFluor488-labeled oxLDL mixed with 40 mg/L unlabeled oxLDL) was added for a further 5 h. Optimal incubation times were determined in time-course experiments (Supplementary Fig. S2). Uptake of oxLDL was doubled after 5 h compared to control; therefore oxLDL was added for 5 h after 24 h incubation with the named compounds. Subsequently, cells were washed with PBS containing Ca^{2+} and Mg^{2+} and detached with lidocaine–EDTA solution. After centrifugation (250 *g*, 8 min, 4 °C) cells were washed with PBS (Ca^{2+} and Mg^{2+}). After staining with 7-AAD, fluorescence of cells was measured on a FACSCalibur, and only live cells were taken. Data were analyzed with FlowJo software 9.

CD36 blocking experiments

CD36 was blocked with human CD36 blocking antibody for 30 min (Antibodies-Online, Aachen, Germany) after incubation of THP-1 macrophages with α-TOH and its metabolites. Cells were then prepared using concentrations and incubation conditions as used for CD36 protein expression analyses. For detecting changes in modified lipoprotein uptake in the presence of α-TOH and its long-chain metabolites one-fifth of applied lipoproteins was labeled with AlexaFluor488 (Invitrogen). Subsequently the cells were harvested and washed twice as described above and analyzed with a FACSCalibur and FlowJo software.

Nile red staining of neutral lipids

Mature THP-1 macrophages were incubated with α-13′-OH and α-13′-COOH for 24 h at the concentrations indicated before addition of 50 mg/L oxLDL to the cell culture medium. After 48 h overall incubation the cells were washed twice with PBS, harvested with Accutase I, washed, and resuspended in PBS. Nile red (Sigma–Aldrich) was added to a final concentration of 1 mg/L, and the cells were incubated for 10 min at room temperature in the dark. After being washed with PBS twice, the cells were resuspended in PBS and measured on a FACSCanto II and analyzed using FlowJo software [25].

Quantification of phagocytosis

THP-1 macrophages were incubated with α-13′-COOH and α-13′-OH for 24 h at the concentrations indicated. To analyze phagocytic activity fluorescent microbeads (35 microbeads/cell; Polysciences, Warrington, PA, USA) were added to the cells. To optimize experimental conditions time-course experiments were performed (Supplementary Fig. S6). This revealed incubation of cells for 24 h with microbeads as the best condition. After three washes the macrophages were harvested with lidocaine–EDTA solution and fluorescence was measured with a FACSCalibur or FACSCanto II and analyzed by FlowJo software.

Semisynthesis of α-LCMs

Chemicals and analytics

All chemicals were used as received from the supplier. Antimycin A, PtO_2, $LiAlH_4$, and $SnCl_2$ were from Sigma–Aldrich. Diethyl ether, methanol, and chloroform were from Roth. *Garcinia kola* seeds were a gift from AnalytiCon Discovery (Potsdam, Germany).

Isolation of garcinoic acid and metabolite synthesis

Isolation of the educt garcinoic acid from the African bitter nut *G. kola* and synthesis of the long-chain metabolites were performed as described [26,27].

Plasma analyses

The blood of healthy male middle-aged (39 years), nonsmoking, normolipidemic volunteers with BMI <25, normal blood pressure, balanced diet with no additional vitamin E supplementation, and no medications known to affect xenobiotic metabolism was used. Written consent to participate in blood withdrawal and analysis of their serum samples was obtained. Blood sampling was carried out on fasted volunteers. Samples were prepared using serum Monovettes (Sarstedt, Nümbrecht, Germany) by centrifugation (2000 *g*) of blood for 10 min at 20 °C. Samples were then maintained at −80 °C until analysis.

For extraction, 500 μl of serum was gently mixed with 25 μl of 10 mg/ml ascorbic acid, 1500 IU of *Escherichia coli* β-glucuronidase type IX-A, and 26 IU/ml *Helix promatia* H-1 sulfatase (both Sigma–Aldrich). Both enzymes were prepared in 200 μl of 0.25 M sodium acetate puffer (pH 6.0). Next the samples were incubated for 30 min at 34 °C under a N_2 atmosphere. Extraction was performed twice using 5 ml of hexane/dichlormethane (ratio 5:2; Sigma–Aldrich) containing 1% butylated hydroxytoluene (Sigma–Aldrich). After mixing for 1 min at room temperature, the samples were centrifuged (2000 *g*, 15 min, 10 °C). The upper organic layers were collected in glass tubes, dried under a N_2 stream, and resuspended in 50 μl methanol (Sigma–Aldrich) for analysis by an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent, Palo Alto, CA, USA) equipped with an Agilent Dual Jet Stream Technology ESI source operating in positive and negative ion mode (fragmentor 300 V, Vcap 4000 V). α-13′-COOH was analyzed by the positive acquisition method and TIC mode with $[M+H]^+$ protonated molecules (*m/z* 461.4). Chromatographic separation was achieved with a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μmol/L) maintained at 35 °C and a flow rate of 0.2 ml/min with a binary gradient of solvent A (H_2O containing 0.1% formic acid) and solvent B (methanol). The gradient was with 80% solvent B at the beginning, 3 min with 99% solvent B, and 15 min with 99% solvent B. Injection volume was 3 μl. Spectrometric data were recorded in the range of 100–1000 *m/z* at 2 GHz. MS/MS spectra were obtained with a collisional energy of 40 V. The curtain gas used was nitrogen. The LC/MS Q-TOF system was governed via Agilent MassHunter Workstation software. For calibration external standard curves of α-13′-COOH in the range of 1 nmol/L to 1 μmol/L were performed.

Statistics

Statistical analyses were performed using Microsoft Excel. To test for statistical significance paired Student's *t* tests were performed.

Results

Metabolism of vitamin E isomers (mainly tocopherols and tocotrienols) has been well established, but little is known about extrahepatic occurrence and physiological relevance of the intermediate α-LCMs because these were not available as pure compounds until recently [27], and no methods for analytic measurement have been described. We therefore established a procedure for reliable detection of α-LCMs in serum and used the pure compounds for functional studies.

α-13′-COOH is present in human serum

γ- and δ-LCMs have been detected in rat liver and in the human cell line A549 [28], and α-LCMs, α-13′-OH and α-13′-COOH, have been identified in human liver cells and feces but not yet in human serum [28,29]. We wondered whether α-13′-OH and α-13′-COOH can be found in human serum. We therefore used pure α-13′-OH and α-13′-COOH obtained semisynthetically as standards for LC/MS Q-TOF analyses of serum obtained from a healthy male volunteer. These analyses revealed considerable amounts of the long-chain metabolite α-13′-COOH in isolated human serum (Fig. 1), whereas α-13′-OH was not detectable with our procedure. Our present procedure allows for qualitative analyses of α-13′-OH and α-13′-COOH but not quantitative measurements. Nevertheless, our results provide first evidence for the systemic bioavailability of α-LCMs.

α-TOH and its α-LCMs differentially affect cell viability

The physiological role of circulating α-LCMs is not known; we therefore performed in vitro studies. Because previous studies revealed apoptotic effects of the α-LCMs [27], we first determined EC_{50} values for α-13′-COOH and α-13′-OH in our macrophage model by sulforhodamine B assays. By using concentrations up to 100 μmol/L no EC_{50} value for α-13′-OH was defined. In contrast, α-13′-COOH revealed an EC_{50} value of 7.4 ± 1.5 μmol/L. In previous studies α-TOH concentrations between 10 and 100 μmol/L have been applied to human and murine macrophages [30,31]. Sulforhodamine B assays confirmed that α-TOH can be used in concentrations up to 100 μmol/L without any cytotoxic effects. Based on these results we consistently used noncytotoxic concentrations of 100 μmol/L α-TOH, 10 μmol/L α-13′-OH, and 5 μmol/L α-13′-COOH for all further investigations. We decided to investigate α-13′-OH in addition to α-13′-COOH in all experiments to get first insights into how important the grade of oxidation of the side chain is for the biological function of the α-LCMs.

CD36 expression is induced by α-LCMs

Studies in vitro have shown that α-TOH prevents oxLDL-induced foam cell formation of smooth muscle cells and macrophages by downregulating CD36 [32]. We therefore investigated the effect of the α-LCMs on macrophage foam cell formation.

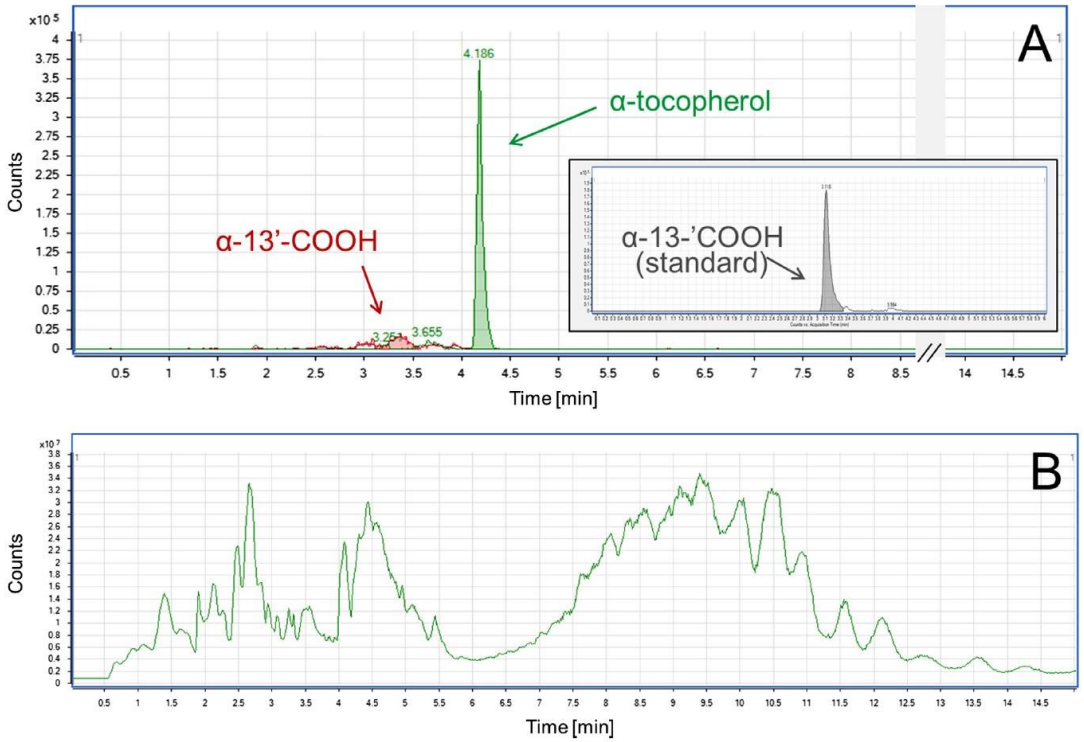

Fig. 1. Detection of α-13′-COOH in human serum. (A) Overlay of the chromatograms of α-13′-COOH and α-TOH in serum obtained from a male volunteer. The chromatogram of the α-13′-COOH standard is shown within the box. The metabolite α-13′-COOH was analyzed by positive acquisition method and TIC mode with $[M+H]^+$ protonated molecules (m/z 461.4). (B) Full mass chromatogram of the serum analyzed in TIC mode and shown in (A). Analyses were performed by LC/MS Q-TOF.

Compared with solvent-treated control THP-1 macrophages, treatment with 10 μmol/L α-13′-OH and 5 μmol/L α-13′-COOH significantly increased CD36 mRNA levels 4.3- and 4.5-fold, respectively ($p < 0.001$; Fig. 2). In contrast to its α-LCMs, α-TOH significantly reduced CD36 mRNA expression to 0.5-fold compared to untreated controls ($p < 0.05$). Further experiments revealed that the effects of α-13′-OH and α-13′-COOH on CD36 mRNA expression were dose-dependent (Supplementary Fig. S3).

Next, flow cytometric analyses were performed to confirm changes in CD36 mRNA at the protein level. As reported previously by others [13], α-TOH significantly decreased CD36 protein by about 11 ± 7% after 24 h of treatment compared to control macrophages ($p < 0.05$). In contrast, CD36 protein levels were significantly increased by α-13′-OH and α-13′-COOH to 144 ± 18 and 170 ± 29%, respectively, after 24 h of incubation compared to controls ($p < 0.05$; Fig. 3A). To confirm the biological relevance of these observations, experiments were reproduced using primary human monocyte-derived macrophages. As shown in Fig. 3B, significant changes in CD36 protein levels by α-13′-OH and α-13′-COOH were observed that were similar to those in THP-1 macrophages. In primary cells, α-13′-OH and α-13′-COOH increased CD36 protein levels up to 133 ± 9 and 137 ± 28%, respectively, compared with untreated cells ($p < 0.01$, $p < 0.05$).

CD36 protein is increased by α-LCMs in the presence of lipoproteins

The scavenger receptor CD36 binds oxLDL and mediates uptake of this modified lipoprotein [7]. As a consequence, oxLDL induces expression of CD36 and hence increases its own uptake [33]. We therefore wanted to know how the regulatory effects of oxLDL are influenced by α-13′-OH and α-13′-COOH. To avoid saturation during lipid loading with oxLDL, we first determined appropriate concentration and incubation time for oxLDL. As a result macrophages were incubated with 50 mg/L oxLDL for 24 h, as a state of early foam cell formation, and for 48 h after a preincubation period in which cells were cultured either in the presence or in the absence of α-TOH, α-13′-OH, or α-13′-COOH.

After preincubation with DMSO (controls), 100 μmol/L α-TOH, 10 μmol/L α-13′-OH, or 5 μmol/L α-13′-COOH for 24 h, 50 mg/L of lipoproteins was added for another 24 h. OxLDL in contrast to nLDL increased CD36 expression significantly (43 ± 11%, $p < 0.001$; Fig. 4). α-TOH decreased CD36 expression significantly by 17 ± 8% in the presence of oxLDL and 15 ± 13.6% in the presence of nLDL compared to cells incubated with lipoproteins but in the absence of α-TOH ($p < 0.05$ and $p < 0.01$), whereas α-13′-OH and α-13′-COOH significantly enhanced CD36 expression (oxLDL, 55 ± 18 and 75 ± 18%, respectively, $p < 0.01$; nLDL, 61 ± 26 and 70 ± 30%,

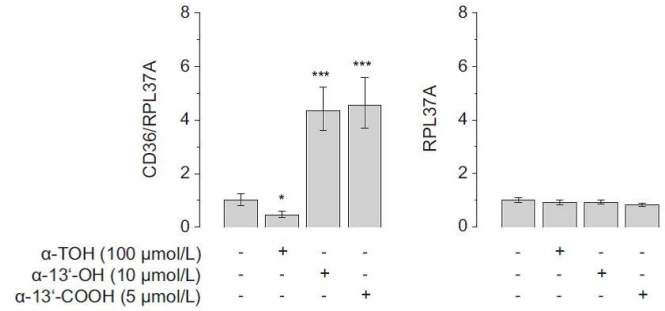

Fig. 2. Expression of human CD36 mRNA is reduced by α-TOH and induced by α-13′-OH and α-13′-COOH in THP-1 macrophages. 3.3×10^6 matured THP-1 cells per 25-cm^2 flask were treated with 100 μmol/L α-TOH, 10 μmol/L α-13′-OH, or 5 μmol/L α-13′-COOH for 24 h. Levels of CD36 mRNA were measured by RT-qPCR and normalized to RPL37A mRNA. Long-chain metabolites, α-13′-OH and α-13′-COOH, significantly increased CD36 mRNA levels by 4.3- and 4.5-fold, respectively (left). CD36 mRNA expression was significantly reduced by up to 0.5-fold by α-TOH compared to untreated control cells (left). Expression of RPL37A remained unchanged under all conditions (right). Error bars display calculated maximum and minimum expression levels representing SEM expression levels of three independent biological experiments, each measured in two technical replicates. *$p < 0.05$; ***$p < 0.001$ (vs no treatment).

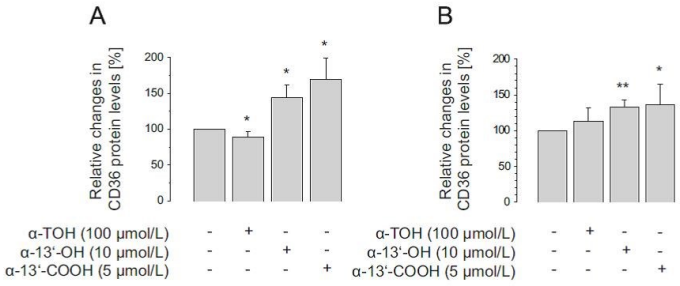

Fig. 3. Expression of human CD36 protein in THP-1 and primary macrophages is induced by α-LCMs. CD36 protein levels were measured by flow cytometry in (A) 1.5×10^6 THP-1 macrophages and (B) 2×10^5 PBMC-derived primary macrophages after incubation with 100 μmol/L α-TOH, 10 μmol/L α-13′-OH, or 5 μmol/L α-13′-COOH for 24 h. Untreated cells served as controls. (A) In THP-1 macrophages, α-TOH significantly decreased CD36 protein expression levels by 11 ± 7%, whereas expression of CD36 was significantly induced by the metabolites α-13′-OH and α-13′-COOH by 144 ± 18 and 170 ± 29%, respectively. (B) In primary macrophages treatment with α-TOH showed no significant effect on CD36 protein compared to control macrophages, whereas treatment with α-13′-OH and α-13′-COOH induced CD36 protein levels to 133 ± 9 and 137 ± 28%, respectively, compared to controls. Histogram bars indicate means ± SD of four to seven independent experiments (A) and of five normolipidemic volunteers (two female and three male donors). *$p < 0.05$; **$p < 0.01$ (vs no treatment).

respectively, $p < 0.01$). Interestingly, induction of CD36 by oxLDL was more pronounced in the presence of α-LCMs compared to cells treated with α-LCMs in the presence of nLDL ($p < 0.01$ for both compounds).

α-LCMs impair oxLDL uptake in THP-1 macrophages

Kinetics of oxLDL uptake were determined in time-course experiments. Cells were preincubated for 24 h with 10 μmol/L α-13′-OH and 5 μmol/L α-13′-COOH before oxLDL was added for 0, 1, 2, 4, 6, 8, 12, and 24 h. These experiments revealed that the lowering effect of α-LCMs on oxLDL uptake increased over time (Supplementary Fig. S4A). For further experiments 5 h of oxLDL incubation was chosen. Preincubation with α-13′-OH and α-13′-COOH for 24 h decreased oxLDL uptake by 18 ± 10 and by 19 ± 8%, respectively, compared to oxLDL uptake in the absence of any compound ($p < 0.05$ and $p < 0.01$, respectively; Supplementary Fig. S4B). To investigate whether uptake of oxLDL is mediated by CD36 the uptake of AlexaFluor488-labeled oxLDL in the presence and absence of a CD36 blocking antibody was investigated. OxLDL uptake by THP-1 macrophages was partly inhibited by CD36 blocking antibody as expected (23 ± 11%, $p < 0.001$; Supplementary Fig. S5). The blocking antibody did not affect the LCM-mediated decrease in oxLDL uptake. All lowering effects were significant compared to the lipoprotein uptake by control cells cultured in the absence of compounds or antibody. We therefore assume that the LCM-mediated decrease in the uptake of oxLDL occurs independent of CD36.

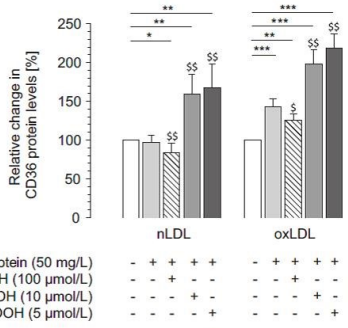

Fig. 4. α-LCMs augment induction of CD36 protein levels by oxLDL. After preincubating 0.5×10^6 THP-1 macrophages per well on a 24-well plate with 100 μmol/L α-TOH, 10 μmol/L α-13′-OH, or 5 μmol/L α-13′-COOH for 24 h, 50 mg/L oxLDL or nLDL was added to the culture medium for another 24 h. OxLDL increased CD36 protein expression significantly (143 ± 11%), whereas nLDL had no effect. Induction of CD36 protein levels by oxLDL was significantly inhibited in the presence of α-TOH by 17 ± 8%. CD36 protein levels were reduced in nLDL-treated cells by 15 ± 14% in the presence of α-TOH compared to untreated control cells and cells cultured in the absence of nLDL and α-TOH. By contrast, α-13′-OH and α-13′-COOH significantly enhanced CD36 expression in cells incubated with nLDL (61 ± 26 and 70 ± 30%, respectively) and further induced expression of CD36 in oxLDL-treated cells (55 ± 18 and 75 ± 18%, respectively). Histogram bars indicate means ± SD of five independent biological experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (vs corresponding no treatment control); $^{\$}p < 0.05$; $^{\$\$}p < 0.01$ (vs treatment with either lipoprotein only).

Uptake of oxLDL by human primary macrophages is decreased by α-13′-OH and α-13′-COOH

The lowering effect of the α-LCMs on oxLDL uptake was unexpected because CD36 was upregulated. We therefore decided to confirm these results in human monocyte-derived macrophages obtained from blood of healthy volunteers. Uptake of oxLDL by primary macrophages was significant ($p < 0.001$), whereas α-13′-OH and α-13′-COOH significantly impaired the uptake of oxLDL (Fig. 5A). A 24-h preincubation with α-13′-OH led to a significant decrease of 24 ± 6% ($p < 0.01$) compared to oxLDL-loaded control cells. α-13′-COOH reduced oxLDL uptake significantly by 20 ± 5% compared to control cells loaded with oxLDL only ($p < 0.01$).

α-LCMs decrease accumulation of neutral lipids in macrophages

To elucidate whether the α-LCMs mediate the accumulation of neutral lipids in macrophages we performed flow cytometric analyses of Nile red-stained cells. OxLDL incubation of THP-1 macrophages led to a significant 28 ± 13% increase in neutral lipids compared to unloaded control cells ($p < 0.01$; Fig. 5B). Pretreatment with the α-LCMs before the addition of oxLDL resulted in reduced accumulation of neutral lipids significantly,

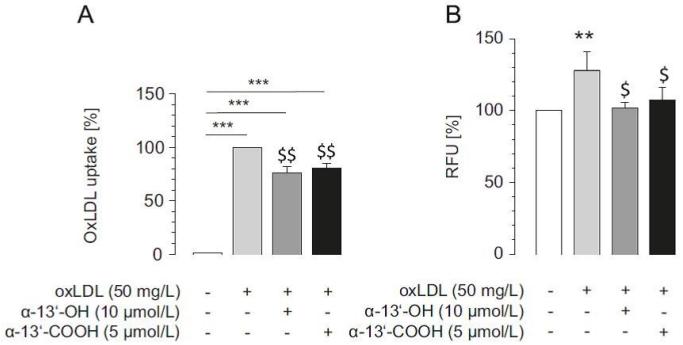

Fig. 5. Uptake of oxLDL and accumulation of neutral lipids by human macrophages is decreased by the α-LCMs. (A) OxLDL uptake was measured by flow cytometry using a mixture of 10 mg/L AlexaFluor488-labeled oxLDL and 40 mg/L unlabeled oxLDL. Peripheral blood mononuclear cells (2.0×10^6/well on a 24-well plate) obtained from healthy donors take up oxLDL significantly. A 24-h preincubation with α-13′-OH led to a significant decrease by 24 ± 6% compared to oxLDL-loaded cells. Similarly, oxLDL uptake was significantly decreased by α-13′-COOH by 20 ± 5% compared to controls loaded with oxLDL only. (B) Relative accumulation of neutral lipids in human THP-1 macrophages (0.5×10^6/well on a 24-well plate) as a result of oxLDL loading was detected by measuring Nile red staining using flow cytometry. OxLDL incubation of mature human macrophages leads to a significant increase in cellular neutral lipid content of 28 ± 13% compared to untreated control cells. Pretreatment of THP-1 macrophages with α-13′-OH or α-13′-COOH significantly reduced oxLDL-induced accumulation of neutral lipids by 17 ± 15, 26 ± 4, and 21 ± 9%, respectively. Histogram bars indicate means ± SD of four independent experiments. $**p < 0.01$; $***p < 0.001$ (vs no treatment); $^{\$}p < 0.05$; $^{\$\$}p < 0.01$ (vs oxLDL only).

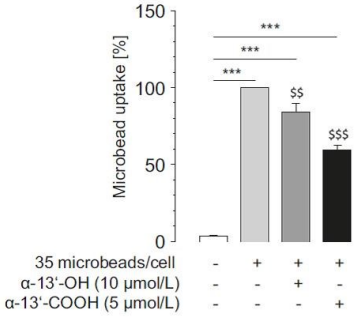

Fig. 6. α-13′-OH and α-13′-COOH inhibit phagocytosis in macrophages. Phagocytic uptake of fluorescence-labeled microbeads was measured by flow cytometry. 0.5×10^6 THP-1 macrophages per well on a 24-well plate were cultured in the presence or absence of 10 μmol/L α-13′-OH or 5 μmol/L α-13′-COOH for 24 h followed by an additional incubation with fluorescence-labeled microbeads for another 24 h. Phagocytic activity was significantly decreased by α-13′-OH and α-13′-COOH by 16 ± 6 and 41 ± 3%, respectively, compared to cells loaded with microbeads in the absence of the metabolites. Bars indicate means ± SD of six independent biological replicates. ***$p < 0.001$ (vs controls in the absence of compounds and microbeads); $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ (vs cells incubated with microbeads in the absence of metabolites).

for α-13′-OH by 26 ± 4% and for α-13′-COOH by 21 ± 9% ($p < 0.05$), respectively.

Long-chain metabolites of α-TOH induce phagocytosis

The apparently contradictory results of increased CD36 expression and decreased uptake of oxLDL let us reason that other oxLDL uptake pathways are modulated by α-13′-OH and α-13′-COOH. Phagocytosis also contributes to the uptake of oxLDL by macrophages [34]. We thus investigated whether α-13′-OH and α-13′-COOH modulate phagocytosis in macrophages. To establish appropriate incubation conditions for fluorescence-labeled microbead uptake assays time-course experiments were performed (Supplementary Fig. S6). After 24 h of preincubation with the α-LCMs, fluorescence-labeled microbeads were added to the culture medium for various times (Supplementary Fig. S6). Differences in phagocytic activity compared to control were observed after 1, 2, 4, 6, 8, 12, and 24 h and were reproducible up to 24 h of incubation with microbeads. We therefore performed assays with THP-1 macrophages cultured in the presence or absence of 10 μmol/L α-13′-OH or 5 μmol/L α-13′-COOH for 24 h followed by 24 h of incubation with labeled microbeads (Fig. 6). Using these conditions we found that α-13′-OH decreased phagocytotic activity significantly by 16 ± 6% ($p < 0.01$), whereas α-13′-COOH impaired phagocytosis significantly, by actually 41 ± 3% ($p < 0.001$) compared to cells loaded with microbeads in the absence of the metabolites.

Discussion and conclusions

Oxidative modification of LDL and uncontrolled uptake of oxLDL by macrophages in the arterial wall via scavenger receptors are key events in atherogenesis [35]. α-TOH, the major isomer of vitamin E, is a potent antioxidant preventing oxidation of LDL in vitro [32]. Moreover, recent studies in vitro suggest that α-TOH exhibits antiatherogenic properties by directly mediating cell signaling and regulating gene expression. For example, α-TOH is an inhibitor of platelet aggregation, suppresses production of nitric oxide and superoxide, and decreases release of proinflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor α [36]. Further, α-TOH inhibits uptake of oxLDL by diminishing scavenger receptor CD36 expression in human macrophages and aortic smooth muscle cells [13,31], thereby inhibiting their transformation into foam cells. Consequently, α-TOH is widely used as a dietary supplement for preventing cardiovascular complications [37]. Studies in humans, however, revealed contradictory results with respect to the ability of α-TOH to prevent cardiovascular complications [38].

Considering the results of the most important intervention studies (e.g., HOPE, GISSI, PPP), vitamin E failed to prevent cardiovascular death [39]. Widely discussed reasons for the poor clinical outcomes are the selection of volunteers, supplementation dose, and time period or rather application form of vitamin E with respect to its bioavailability. Further, most of the intervention studies concentrate on secondary prevention in patients with already existing vascular diseases. As an alternative explanation for its failure it has been suggested that vitamin E supplementation for primary prevention may be more effective for inhibiting the early stages of atherosclerosis [39]. However, this has not yet been confirmed. These findings in humans raise the question whether α-TOH in vivo exhibits modes of action different from those found in vitro.

In humans, α-TOH is metabolized in the liver by side-chain truncation initiated by CYP3A4- and/or CYP4F2-dependent ω-hydroxylation, which results in the formation of the alcohol metabolite α-13′-OH (Supplementary Fig. S1). Subsequent α-oxidation in peroxisomes forms the acid metabolite α-13′-COOH, which is rapidly metabolized by two steps of β-oxidation in peroxisomes and three further steps in mitochondria to the metabolic end-product α-CEHC, which is released via urine [9]. Recently, several tocopherol α-LCMs, but not α-13′-OH and α-13′-COOH, have been found by HPLC/EC analyses in serum of mice and humans after supplementation [17]. We wondered whether α-13′-OH and α-13′-COOH can be detected in human serum using LC/MS, and indeed we found α-13′-COOH at nanomolar scale in the serum of a nonsupplemented individual. But we were not able to detect the metabolic precursor of α-13′-COOH, namely α-13′-OH. We assume that the catabolism of α-13′-OH to α-13′-COOH occurs rapidly so that the intermediate metabolite cannot be detected in serum; alternatively, the metabolite α-13′-OH may not be released into blood. However, our findings do not provide hints on the tissue origin of serum α-13′-COOH but provide clear evidence for systemic bioavailability of this LCM.

Little information on the physiological properties of α-LCMs is available. We therefore asked the question whether α-13′-OH and α-13′-COOH exhibit a regulatory capacity similar to α-TOH. Recent studies demonstrate that α-LCMs of α- and δ-TOH exhibit proapoptotic properties and provoke mitochondrial dysfunction in HepG2 cells [27]. Further, Jiang et al. [40] showed that δ-13′-COOH and γ-13′-COOH inhibit COX2-catalyzed production of prostaglandin E_2 in IL-1β-stimulated human lung adenocarcinoma A549 cells. In our study we were interested in whether the α-LCMs regulate oxLDL uptake and macrophage foam cell formation. Macrophages express scavenger receptor CD36, which accounts for the major proportion of uptake of modified LDL species, such as oxLDL [7], and α-TOH is known to downregulate CD36 expression, thus preventing oxLDL-induced foam cell formation [13]. Our study confirms downregulation of CD36 by α-TOH in macrophages (Figs. 2 and 3A) and shows furthermore that α-LCMs exhibit opposing effects in macrophages (Fig. 3). More importantly, upregulation of CD36 in macrophages by α-13′-OH and α-13′-COOH took place in the presence of native LDL and was even more pronounced in the presence of oxLDL (Fig. 4). This cumulative effect of the α-LCMs is in contrast to the diminished oxLDL-mediated induction of CD36 by α-TOH in macrophages found here (Fig. 4) and reported by others [30,31].

Based on our findings we hypothesized that α-LCMs enhance oxLDL uptake and hence macrophage foam cell formation. However, α-13′-OH and α-13′-COOH unexpectedly decreased uptake of oxLDL in macrophages although CD36 levels were increased (Fig. 3A). Experiments with blocking antibodies confirmed the involvement of CD36 in oxLDL uptake (Supplementary Fig. S5), but revealed no cumulative inhibitory effect on oxLDL uptake in addition to that of α-LCMs, suggesting that downregulation of oxLDL uptake by α-13′-OH and α-13′-COOH takes place at least in part independent of CD36.

The contradictory and maybe compensatory upregulation of CD36 and the decrease in oxLDL uptake provide evidence that other uptake pathways are modulated by α-13′-OH and α-13′-COOH. Apart from scavenger receptor-mediated uptake, oxLDL particles can be internalized by phagocytosis [41]. Interestingly, α-13′-OH and α-13′-COOH decrease phagocytic activity of macrophages (Fig. 6), thus again exhibiting functional characteristics contrary to those of α-TOH, as Izgut-Uysal et al. [41] found that α-TOH increases phagocytic activity of peritoneal macrophages. As a consequence of reduced oxLDL uptake, α-TOH and its metabolites α-13′-OH and α-13′-COOH antagonize oxLDL-induced transformation of macrophages into foam cells.

In our studies we have used α-TOH in concentrations of 100 μM, although such levels usually cannot be achieved in humans even during oral supplementation with α-TOH. However, in previous cell culture studies, which have shown that α-TOH decreases CD36 expression in human monocyte-derived macrophages, α-TOH concentrations of 100 μM have been used [30,31]. We have used α-TOH here as a control to demonstrate the validity of our in vitro model. We therefore used the same concentrations that have been used in published studies to exert comparable effects of α-TOH on CD36 expression in order to provide results comparable to previously published data. We also have to concede that our results were obtained with micromolar concentrations of the α-LCMs, i.e., 5 μM for α-13′-COOH and 10 μM for α-13′-OH, and we are aware that these concentrations are higher than the estimated physiological concentrations of the α-LCMs in human serum. On the other hand this is a common problem of in vitro studies, in which metabolite and drug concentrations of even 1 to 10 μM are often required to exert reliable effects [42–44]. Furthermore, at present we do not know the cellular concentrations of the α-LCMs that can be achieved in vitro and in vivo, so that uptake experiments have to be performed in future studies to better understand the physiological relevance of the findings outlined here.

However, we do not know whether cellular uptake of the α-LCMs is required to affect cellular signaling and processes. Even in the case in which the metabolite is not taken up by cells, binding of the α-LCMs to specific cell surface receptors may be responsible for mediating outside-in signaling. Further studies are therefore required to unravel the exact mode of action of the α-LCMs by identifying the involved receptors and mediators.

Overall, our study shows that α-TOH and its metabolites α-13′-OH and α-13′-COOH modulate macrophage foam cell formation via different pathways. Hence, our results provide a new perception on how vitamin E and its physiological α-LCMs may regulate processes in vivo. However, our data also show that the mode of action of α-TOH and its metabolites α-13′-OH and α-13′-COOH may be much more complex in vivo than expected from experiments in vitro and that further studies are required to elucidate the physiological role and exact modes of action (e.g., on involved signaling pathways) of the metabolites and their contribution to disease processes such as atherosclerosis. Studies on the physiological function and clinical importance of the α-LCMs have been initiated.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2013.11.009.

References

[1] Cullen, P.; Rauterberg, J.; Lorkowski, S. The pathogenesis of atherosclerosis. *Handb. Exp. Pharmacol.* **170**:3–70; 2005.
[2] Harel, M.; Aharoni, A.; Gaidukov, L.; Brumshtein, B.; Khersonsky, O.; Meged, R.; Dvir, H.; Ravelli, R. B.; McCarthy, A.; Toker, L.; Silman, I.; Sussman, J. L.; Tawfik, D. S. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nat. Struct. Mol. Biol.* **11**:412–419; 2004.
[3] Ricardo, S. D.; van Goor, H.; Eddy, A. A. Macrophage diversity in renal injury and repair. *J. Clin. Invest.* **118**:3522–3530; 2008.
[4] Goldstein, J. L.; Ho, Y. K.; Basu, S. K.; Brown, M. S. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA* **76**:333–-337; 1979.
[5] Schwartz, C. J.; Valente, A. J.; Sprague, E. A.; Kelley, J. L.; Nerem, R. M. The pathogenesis of atherosclerosis: an overview. *Clin. Cardiol.* **14**:I1–16; 1991.
[6] Ghosh, S.; Zhao, B.; Bie, J.; Song, J. Macrophage cholesteryl ester mobilization and atherosclerosis. *Vasc. Pharmacol.* **52**:1–10; 2010.
[7] Collot-Teixeira, S.; Martin, J.; McDennott-Roe, C.; Poston, R.; McGregor, J. L. CD36 and macrophages in atherosclerosis. *Cardiovasc. Res.* **75**:468–477; 2007.
[8] Erwig, L. P.; Henson, P. M. Clearance of apoptotic cells by phagocytes. *Cell Death Differ.* **15**:243–250; 2008.
[9] Mustacich, D. J.; Leonard, S. W.; Patel, N. K.; Traber, M. G. α-Tocopherol β-oxidation localized to rat liver mitochondria. *Free Radic. Biol. Med.* **48**:73–81; 2010.
[10] Tucker, J. M.; Townsend, D. M. Alpha-tocopherol: roles in prevention and therapy of human disease. *Biomed. Pharmacother.* **59**:380–387; 2005.
[11] Landes, N.; Pfluger, P.; Kluth, D.; Birringer, M.; Ruhl, R.; Bol, G. F.; Glatt, H.; Brigelius-Flohe, R. Vitamin E activates gene expression via the pregnane X receptor. *Biochem. Pharmacol.* **65**:269–273; 2003.
[12] Farley, S. M.; Leonard, S. W.; Taylor, A. W.; Birringer, M.; Edson, K. Z.; Rettie, A. E.; Traber, M. G. ω-Hydroxylation of phylloquinone by CYP4F2 is not increased by α-tocopherol. *Mol. Nutr. Food Res.* **57**:1785–1793; 2013.
[13] Ricciarelli, R.; Zingg, J. M.; Azzi, A. Vitamin E reduces the uptake of oxidized LDL by inhibiting CD36 scavenger receptor expression in cultured aortic smooth muscle cells. *Circulation* **102**:82–87; 2000.
[14] Campbell, S. E.; Stone, W. L.; Whaley, S. G.; Qui, M.; Krishnan, K. Gamma (γ) tocopherol upregulates peroxisome proliferator activated receptor (PPAR) gamma (γ) expression in SW 480 human colon cancer cell lines. *BMC Cancer* **3**:25; 2003.
[15] Azzi, A.; Gysin, R.; Kempna, P.; Munteanu, A.; Negis, Y.; Villacorta, L.; Visarius, T.; Zingg, J. M. Vitamin E mediates cell signaling and regulation of gene expression. *Ann. N. Y. Acad. Sci.* **1031**:86–95; 2004.
[16] Birringer, M.; Drogan, D.; Brigelius-Flohe, R. Tocopherols are metabolized in HepG2 cells by side chain ω-oxidation and consecutive β-oxidation. *Free Radic. Biol. Med.* **31**:226–232; 2001.
[17] Zhao, Y.; Lee, M. J.; Cheung, C.; Ju, J. H.; Chen, Y. K.; Liu, B.; Hu, L. Q.; Yang, C. S. Analysis of multiple metabolites of tocopherols and tocotrienols in mice and humans. *J. Agric. Food Chem.* **58**:4844–4852; 2010.
[18] Schultz, M.; Leist, M.; Petrzika, M.; Gassmann, B.; Brigelius-Flohe, R. Novel urinary metabolite of alpha-tocopherol, 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman, as an indicator of an adequate vitamin E supply? *Am. J. Clin. Nutr.* **62**:1527S–1534S; 1995.
[19] Schnoor, M.; Buers, I.; Sietmann, A.; Brodde, M. F.; Hofnagel, O.; Robenek, H.; Lorkowski, S. Efficient non-viral transfection of THP-1 cells. *J. Immunol. Methods* **344**:109–115; 2009.
[20] Schnoor, M.; Cullen, P.; Lorkowski, J.; Stolle, K.; Robenek, H.; Troyer, D.; Rauterberg, J.; Lorkowski, S. Production of type VI collagen by human macrophages: a new dimension in macrophage functional heterogeneity. *J. Immunol.* **180**:5707–5719; 2008.
[21] Mosig, S.; Rennert, K.; Krause, S.; Kzhyshkowska, J.; Neunubel, K.; Heller, R.; Funke, H. Different functions of monocyte subsets in familial hypercholesterolemia: potential function of $CD14^+$ $CD16^+$ monocytes in detoxification of oxidized LDL. *FASEB J.* **23**:866–874; 2009.

[22] Stolle, K.; Schnoor, M.; Fuellen, G.; Spitzer, M.; Cullen, P.; Lorkowski, S. Cloning, genomic organization, and tissue-specific expression of the RASL11B gene. *Biochim. Biophys. Acta* **1769**:514–524; 2007.
[23] Stolle, K.; Schnoor, M.; Fuellen, G.; Spitzer, M.; Engel, T.; Spener, F.; Cullen, P.; Lorkowski, S. Cloning, cellular localization, genomic organization, and tissue-specific expression of the TGFbeta1-inducible SMAP-5 gene. *Gene* **351**:119–-130; 2005.
[24] Havel, R. J.; Eder, H. A.; Bragdon, J. H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**:1345–1353; 1955.
[25] Halstead, B. W.; Zwickl, C. M.; Morgan, R. E.; Monteith, D. K.; Thomas, C. E.; Bowers, R. K.; Berridge, B. R. A clinical flow cytometric biomarker strategy: validation of peripheral leukocyte phospholipidosis using Nile red. *J. Appl. Toxicol.* **26**:169–177; 2006.
[26] Terashima, K.; Shimamura, T.; Tanabayashi, M.; Aqil, M.; Akinniyi, J. A.; Niwa, M. Constituents of the seeds of Garcinia kola: two new antioxidants, garcinoic acid and garcinal. *Heterocycles* **45**:1559–1566; 1997.
[27] Birringer, M.; Lington, D.; Vertuani, S.; Manfredini, S.; Scharlau, D.; Glei, M.; Ristow, M. Proapoptotic effects of long-chain vitamin E metabolites in HepG2 cells are mediated by oxidative stress. *Free Radic. Biol. Med.* **49**:1315–1322; 2010.
[28] Jiang, Q.; Freiser, H.; Wood, K. V.; Yin, X. Identification and quantitation of novel vitamin E metabolites, sulfated long-chain carboxychromanols, in human A549 cells and in rats. *J. Lipid Res.* **48**:1221–1230; 2007.
[29] Birringer, M.; Pfluger, P.; Kluth, D.; Landes, N.; Brigelius-Flohe, R. Identities and differences in the metabolism of tocotrienols and tocopherols in HepG2 cells. *J. Nutr.* **132**:3113–3118; 2002.
[30] Venugopal, S. K.; Devaraj, S.; Jialal, I. RRR-alpha-tocopherol decreases the expression of the major scavenger receptor, CD36, in human macrophages via inhibition of tyrosine kinase (Tyk2). *Atherosclerosis* **175**:213–220; 2004.
[31] Devaraj, S.; Hugou, I.; Jialal, I. Alpha-tocopherol decreases CD36 expression in human monocyte-derived macrophages. *J. Lipid Res.* **42**:521–527; 2001.
[32] Munteanu, A.; Taddei, M.; Tamburini, I.; Bergamini, E.; Azzi, A.; Zingg, J. M. Antagonistic effects of oxidized low density lipoprotein and alpha-tocopherol on CD36 scavenger receptor expression in monocytes: involvement of protein kinase B and peroxisome proliferator-activated receptor-gamma. *J. Biol. Chem.* **281**:6489–6497; 2006.
[33] Silverstein, R. L.; Li, W.; Park, Y. M.; Rahaman, S. O. Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis. *Trans. Am. Clin. Climatol. Assoc.* **121**:206–220; 2010.
[34] Schrijvers, D. M.; De Meyer, G. R.; Herman, A. G.; Martinet, W. Phagocytosis in atherosclerosis: molecular mechanisms and implications for plaque progression and stability. *Cardiovasc. Res.* **73**:470–480; 2007.
[35] McLaren, J. E.; Michael, D. R.; Guschina, I. A.; Harwood, J. L.; Ramji, D. P. Eicosapentaenoic acid and docosahexaenoic acid regulate modified LDL uptake and macropinocytosis in human macrophages. *Lipids* **46**:1053–1061; 2011.
[36] Azzi, A.; Ricciarelli, R.; Zingg, J. M. Non-antioxidant molecular functions of alpha-tocopherol (vitamin E). *FEBS Lett.* **519**:8–10; 2002.
[37] Azzi, A. The role of alpha-tocopherol in preventing disease. *Eur. J. Nutr.* **43** (**Suppl. 1**); 2004. (I/18-25).
[38] Rodrigo, R.; Guichard, C.; Charles, R. Clinical pharmacology and therapeutic use of antioxidant vitamins. *Fundam. Clin. Pharmacol.* **21**:111–127; 2007.
[39] Vivekananthan, D. P.; Penn, M. S.; Sapp, S. K.; Hsu, A.; Topol, E. J. Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. *Lancet* **361**:2017–2023; 2003.
[40] Jiang, Q.; Yin, X.; Lill, M. A.; Danielson, M. L.; Freiser, H.; Huang, J. Long-chain carboxychromanols, metabolites of vitamin E, are potent inhibitors of cyclooxygenases. *Proc. Natl. Acad. Sci. USA* **105**:20464–20469; 2008.
[41] Izgut-Uysal, V. N.; Tan, R.; Bulbul, M.; Derin, N. Effect of stress-induced lipid peroxidation on functions of rat peritoneal macrophages. *Cell Biol. Int.* **28**:517–521; 2004.
[42] Janowski, B. A.; Willy, P. J.; Devi, T. R.; Falck, J. R.; Mangelsdorf, D. J. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* **383**:728–731; 1996.
[43] Tam, S. P.; Mok, L.; Chimini, G.; Vasa, M.; Deeley, R. G. ABCA1 mediates high-affinity uptake of 25-hydroxycholesterol by membrane vesicles and rapid efflux of oxysterol by intact cells. *Am. J. Physiol. Cell Physiol.* **291**:C490–C502; 2006.
[44] Chinetti, G.; Lestavel, S.; Bocher, V.; Remaley, A. T.; Neve, B.; Torra, I. P.; Teissier, E.; Minnich, A.; Jaye, M.; Duverger, N.; Brewer, H. B.; Fruchart, J. C.; Clavey, V.; Staels, B. PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat. Med.* **7**:53–58; 2001.

3.2 Manuscript II

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Graphical Review

Regulatory metabolites of vitamin E and their putative relevance for atherogenesis

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ABSTRACT

Vitamin E is likely the most important antioxidant in the human diet and α-tocopherol is the most active isomer. α-Tocopherol exhibits anti-oxidative capacity *in vitro*, and inhibits oxidation of LDL. Beside this, α-tocopherol shows anti-inflammatory activity and modulates expression of proteins involved in uptake, transport and degradation of tocopherols, as well as the uptake, storage and export of lipids such as cholesterol. Despite promising anti-atherogenic features *in vitro*, vitamin E failed to be atheroprotective in clinical trials in humans. Recent studies highlight the importance of long-chain metabolites of α-tocopherol, which are formed as catabolic intermediate products in the liver and occur in human plasma. These metabolites modulate inflammatory processes and macrophage foam cell formation via mechanisms different than that of their metabolic precursor α-tocopherol and at lower concentrations. Here we summarize the controversial role of vitamin E as a preventive agent against atherosclerosis and point the attention to recent findings that highlight a role of these long-chain metabolites of vitamin E as a proposed new class of regulatory metabolites. We speculate that the metabolites contribute to physiological as well as pathophysiological processes.

Contents

Introduction

Atherosclerosis is a progressive inflammatory disease characterized by excessive deposition of cholesterol in the arterial wall. Despite intensive therapeutic treatment opportunities the atherosclerotic complications are still the leading cause of death in Western industrialized countries.

Leonardo da Vinci (1452–1519) was probably the first who described the macroscopic changes of atherosclerosis, when he illustrated the lesions in arteries obtained from an elderly man at autopsy. His visionary idea was that the pathological thickening

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Nomenclature

α-13′-OH	α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanol
α-13′-COOH	α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid
α-CEHC	α-carboxyethyl-hydroxychroman
α-LCM	α-tocopherol long-chain metabolites
α-SCM	α-tocopherol short-chain metabolites
α-TOH	α-tocopherol
COX	cyclooxygenase
CYP3A4	cytochrome P450, subfamily IIIA, polypeptide 4
CYP4F2	cytochrome P450, subfamily IVF, polypeptide 2
LDL	low density lipoprotein
oxLDL	oxidized low density lipoprotein

of the arterial wall was due to 'excessive nourishment' from the blood. Many decades later da Vinci's observation was studied in more detail by Carl von Rokitansky (1852) and Rudolf L. K. Virchow (1821–1902). In 1856 Virchow proposed that injury of the endothelium may initiate the disease process of atherosclerosis. Based on this idea, Russell Ross (1929–1999) and John A. Glomset came up in 1973 with the 'response-to-injury' hypothesis which is still generally accepted today in the form of the more generalized concept of endothelial dysfunction as the initial cause of atherosclerosis.

The pioneering work of Virchow and Nikolai N. Anitschkow (1885–1964) provided first evidence for the importance of the deposition of lipids from the blood, in particular cholesterol, in the arterial wall. Their findings formed the basis for the lipid hypothesis which connects plasma cholesterol levels to the development of the disease. In 1951, G. Lyman Duff (1904–1956) and Gardner C. McMillan (1918–2004) formulated the lipid hypothesis in its modern form, which is, despite controversial discussions, still widely accepted today. Since the discovery of the importance of the cholesterol contained in low-density lipoprotein (LDL[2]) particles for the pathogenesis of atherosclerosis, the concept of endothelial dysfunction has become tightly linked to the lipid hypothesis.

Almost 30 years ago the concept originated from work by Daniel Steinberg and Joseph L. Witztum that oxidative stress and the oxidation of LDL particles might contribute to atherosclerosis. The idea came up from the observation that the incubation of macrophages with oxidized LDL (oxLDL[3]) but not with native LDL led to the intracellular accumulation of cholesteryl esters. The idea that oxidative stress is involved in atherogenesis gained much attention and created tremendous excitement to look for oxLDL *in vivo* as well as for different kinds of oxidized lipid species within the particle. Since oxLDL appears in human plasma as well as within the arterial wall it was even a small step to the idea that supplementation with antioxidants may prevent atherosclerosis by inhibiting the formation of oxLDL. This hypothesis appeared to be on solid ground due to epidemiological evidence and the success in several animal studies using a variety of antioxidants. The euphoria of initial success led to clinical trials to validate the hypothesis and natural antioxidants were of particular interest as the expectation was that these natural compounds would have less undesirable effects. Accordingly a number of clinical trials were performed using, for example, vitamin E, which surprisingly have not been overwhelmingly supportive of the hypothesis. An overview on the controversial findings for vitamin E obtained from clinical trials is given in Fig. 1.

In this review, we want to summarize the controversial role of vitamin E as a preventive agent against atherosclerosis and to point the attention to recent findings by our group that highlight a role of long-chain metabolites of vitamin E as a proposed new class of regulatory metabolites and to their potential contribution to atherosclerotic processes.

[2] LDL, low density lipoprotein.
[3] oxLDL, oxidized low density lipoprotein.

Pathogenesis of atherosclerosis

The endothelium covering the arterial walls comprises a physiological and selective barrier, the so-called intima, between blood and the inner layer of the arterial wall. This so-called media is comprised by contractile smooth muscle cells. Pathophysiological stimuli cause endothelial dysfunction triggering inflammatory processes in the vascular wall which result under chronic conditions in extensive morphological changes characterized by intimal thickening, deposition of cholesterol and fibrotic material, loss of elasticity, reduction of vascular lumen, and widening of the vessel diameter [1]. Endothelial dysfunction is thought to be caused by exogenous stimuli, such as environmental factors (e.g., toxicants such as dioxins, PCBs, and pesticides), unhealthy lifestyle (e.g., smoking and physical inactivity) and dietary habits (e.g., high intake of saturated fat). The impact of exogenous factors depends on endogenous local and systemic conditions. Local factors are vessel-associated junctions, bifurcations and curvatures which are responsible for increased shear stress caused by turbulences of the blood stream in these areas, which are thus predestinated for the formation of atherosclerotic lesions [2]. Pro-atherogenic systemic factors are determined either genetically or pathophysiologically, for example, in case of increased LDL and triglyceride plasma levels [3,4] as well as inflammatory conditions [5]. The process of atherosclerosis is outlined and explained in more detail in Fig. 2.

A key event of atherogenesis is the loss of the selective endothelial barrier by endothelial dysfunction which allows, for example, LDL to enter the arterial wall. Once inside the vessel wall, LDL particles become prone to oxidation. The oxidized particles cause damage to the tissue thus triggering a cascade of immune and inflammatory responses. In addition, macrophages, the phagocytic cells of the immune system, are recruited to the affected tissue sites to clear the oxLDL particles. As a consequence oxidized lipids and particularly cholesterol accumulate within the macrophages as these cells are not able to process the oxLDL completely. This causes transformation of the cells into so-called foam cells and ultimately cell death as the excessive accumulation of intracellular lipids is cytotoxic. Death of macrophage foam cells results over time in the extracellular deposition of cholesterol in the arterial wall and the formation of an atheroma. The process of atherosclerosis is outlined and explained in more detail in Fig. 2.

Thus, vitamin E was considered as an anti-atherogenic agent for a long time as prevention of LDL oxidation by providing increased levels of antioxidants would prevent the formation of macrophage foam cells and atheroma, and would dampen the immune and inflammatory response.

Effects of α-Tocopherol on atherogenic processes

Vitamin E is likely the most important lipid antioxidant in the human diet. The term vitamin E comprises a group of eight abundant isomers (α-, β-, γ-, δ-tocopherol and -tocotrienol), that differ by their methylation patterns of the hydroxychromanol ring and saturation of the side-chain. Many *in vitro* studies have been performed with

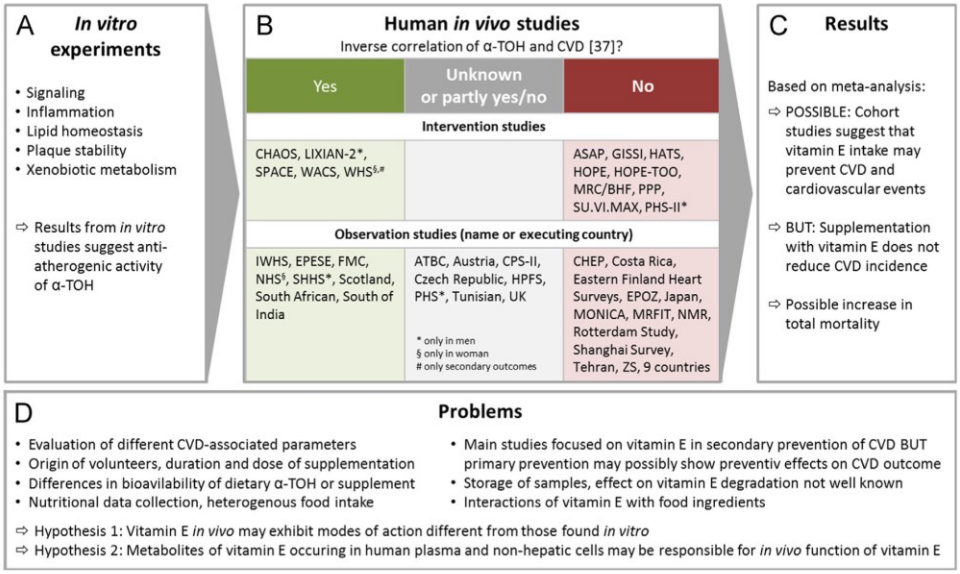

Fig. 1. Effects of α-TOH *in vivo*. Many *in vitro* investigations (A) focused on the identification of α-TOH-regulated signaling pathways and its effects on inflammation, lipid homeostasis, atherosclerotic plaque stability as well as xenobiotic metabolism as key processes. Taken together most of the *in vitro* studies implicated that vitamin E, and particularly α-TOH, may be used to prevent or cure cardiovascular disease (CVD) and related diseases, such as atherosclerosis. Based on very promising studies *in vitro* and with animals several large-scaled human intervention trials were initiated and followed up over years. Unfortunately, the trials revealed controversial results and failed to demonstrate clear invers relations or positive effects of α-TOH supplementation with respect to the prevention of cardiovascular complications [21] (B). Further, α-TOH serum levels did not correlate with cardiovascular outcomes in different cohorts. Although some studies reported promising findings, such as the ‘Nurses' Health Study (NHS) [22] including 87,000 volunteers in which vitamin E supplementation was associated with a lower risk of major coronary disease, other large-scale studies, such as the Heart Outcomes Prevention Evaluation (HOPE) study [23], the SU.VI.MAX study [24], the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI) study [25] did not confirm that vitamin E intake correlates negatively with cardiovascular outcomes. Other studies revealed also contrary results depending on the time of follow-up and the cardiovascular parameter investigated or they showed unclear results (for example, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC) [26–30], the Physicians' Health Study [31], the Health Professionals Follow-up Study (HPFS) [32,33]). In a recent meta-analysis Ye et al. also found no significant inverse correlation for CVD and cardiovascular mortality under vitamin E [34]. Reasons for the poor outcomes of clinical trials are hardly to define. Beside a general failure of vitamin E, other reasons may explain the lack of any cardio-preventive effect. Likely the selection of volunteers, the sizes of cohorts, doses and duration of supplementation or rather the application form of vitamin E with respect to its bioavailability, the food questionnaires and variability of food intake may explain the findings. Furthermore, it cannot be excluded that the interaction of vitamin E with other food ingredients contributes to the lack of its cardio-preventive activity. From a technical point of view, it cannot be excluded that vitamin E in stored sample is chemically modified or degraded so that frozen biological samples are not completely comparable to fresh samples. Another important point refers to the fact that most of the intervention trials concentrated on secondary prevention in patients with already existing CVD. It has been suggested that vitamin E supplementation may be more effectively for inhibiting the early stages of atherosclerosis [35] and should be considered for primary prevention, as recently supported by Meydani et al. emphasizing the beneficial effects of long-term vitamin E supplementation in $Ldlr^{-/-}$ mice under healthy life-style conditions, such as low fat diet [36]. However, this hypothesis has not yet been confirmed. In summary (C), there is no clear evidence that supplementation with vitamin E correlates inversely with CVD incidence. Meta-analyses of observation studies suggest that vitamin E intake may prevent CVD and cardiovascular events [37–39]. Knekt et al. performed a pooled analysis of observation studies with dietary vitamin E intake and supplementation in separate arms and found a significant inverse correlation of intake and CVD events only in the supplementation group [40]. Apart from that meta-analysis of intervention studies provide evidence that supplementation with vitamin E does not reduce CVD incidence [38,41–43]. There are several drawbacks of meta-analysis that should be considered while interpreting these results, such as combination of heterogeneous data sets (regarding quality, statistics and focus within the topic), publication bias as well as criteria for inclusion and exclusion of the meta-analysis. However, it cannot yet be excluded that vitamin E intake is protective at least in some groups of humans against CVD as primary prevention. It is also important to remind that Miller et al. focused in their meta-analysis on some intervention studies, which provided evidence for an increase in all-cause mortality after supplementation with high doses of vitamin E [44]. These findings in humans raise the question whether α-TOH *in vivo* exhibits modes of action different from those found *in vitro*. Possible explanations for the inability of vitamin E to prevent CVD and its complications in clinical trials in humans have been outlined above and are summarized in (D). Abbreviations and references: Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) [26–30], Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) [45], Austria [46], Cambridge Heart Antioxidant Study (CHAOS) [47], Cancer Prevention Study II (CPS-II) [48], Costa Rica [49], Czech Republic [50], Eastern Finland Heart Surveys [51], Epidemiologic Study of Cardiovascular Risk Indicators (EPOZ Study) [52], Established Populations for Epidemiologic Studies of the Elderly (EPESE) [53], Finnish Mobile Clinic Examination Survey (FMC) [54], Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico (GISSI) [25], HDL-Atherosclerosis Treatment Study (HATS) [55], Health Check-up Program (CHEP) [56], Heart Outcomes Prevention Evaluation Study (HOPE) [23], HOPE-The Ongoing Outcomes [HOPE-TOO] [57], Iowa Women's Health Study (IWHS) [58], Japan [59], Lixian dysplasia trial (LIXIAN-2) [60,61], Medical Research Council/British Heart Foundation (MRC/BHF) [62], Multinational MONItoring of trends and determinants in CArdiovascular disease (MONICA) [63], Noninstitutionalized Massachusetts Residents (NMR) [64], Physicians' Health Study (PHS) [31], Physicians' Health Study II (PHS-II) [65], Rotterdam Study [66], Scotland and UK [67], Scottish Heart Health Study (SHHS) [68], Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE) [69], Shanghai Survey [70], South African [71], South of India [72], Supplémentation en Vitamines et Minéraux Antioxydants (SU.VI.MAX) [24], Tehran [73], The Health Professionals Follow-up Study (HPFS) [32,33], The Multiple Risk Factor Intervention Trial (MRFIT) [74], The Nurses' Health Study (NHS) [22], The Primary Prevention Project (PPP) [75], The Zutphen Study (ZS) [76], Tunisian [77], UK [78], Women's Antioxidant Cardiovascular Study (WACS) [79], Women's Health Study (WHS) [80], 9 countries [81]. The name of countries/cities refers to the countries/cities in which the studies have been performed, if no name for the studies is available.

α-tocopherol (α-TOH[4]) which is the most active isomer within the group of vitamin E [6]. *α*-Tocopherol exhibits anti-oxidative capacity *in vitro* [7], and it has been shown to particularly inhibit, for example, the oxidation of LDL. Beside this, α-TOH shows anti-inflammatory features by, for example, inhibiting cyclooxygenase (COX[5]) 2. Next to its anti-inflammatory and anti-oxidative properties, the vitamin E

[4] α-TOH, α-tocopherol.

[5] COX, cyclooxygenase.

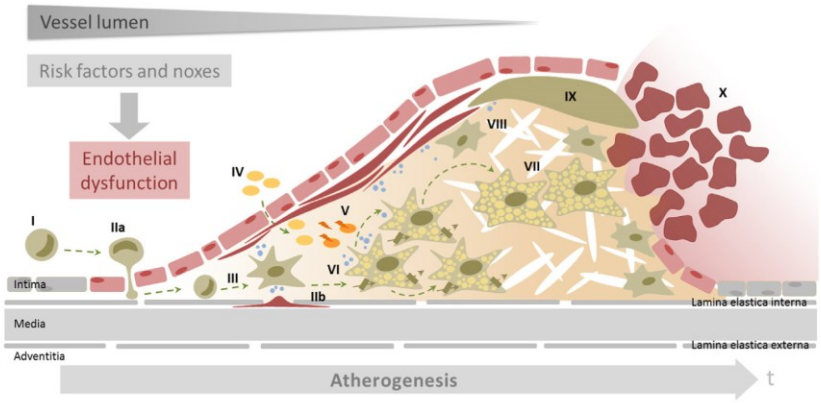

Fig. 2. Key events of atherogenesis. Atherosclerosis is a complex and progressive inflammatory disease characterized by extensive morphological changes of the vascular wall. The arterial wall is composed of the *intima*, formed by endothelial cells (EC), the *media*, comprised of smooth muscle cells (SMC), and the *adventitia*, made of fibroblasts. The intermediate layers of the arterial vessel are the *lamina elastica interna*, connecting the *intima* with the *media*, and the *lamina elastica externa*, the connection between the *media* and the *adventitia*. Key changes of the vascular wall during the progression of atherosclerosis are intimal thickening, extensive extracellular deposition of cholesterol and fibrotic material, loss of elasticity, reduction of the vascular lumen and widening of the arterial diameter [1]. Endothelial dysfunction is caused by exogenous and endogenous noxes and is the initial event in atherosclerosis. The dysfunction of the endothelium is accompanied by up-regulation of adhesion molecules in EC, which promote attachment and recruitment of T-lymphocytes and monocytes from the blood (I) and initiate immigration of monocytes into the subendothelial area of the vessel wall (IIa). Migration of monocytes, T-lymphocytes and SMC from the *media* (IIb) finally results in intimal thickening and fibrosis. Following migration of monocytes through the *intima*, differentiation of these cells into macrophages occurs (III). Macrophages as the phagocytic cells of the immune system play a pivotal role in the progression of atherosclerosis. They actively engage by taking up lipids from oxLDL in a non-controlled manner, by storing large amounts of cholesteryl esters, and by mobilizing cholesterol for reverse cholesterol transport [82,83]. Macrophages also orchestrate the inflammatory process, are responsible for the immigration of SMC from the *media* by releasing chemotactic molecules and proteases, and modulate the fibrotic process. LDL particles diffuse from the blood into the subendothelial space as a consequence of the loss of the endothelial barrier during endothelial dysfunction (IV). In the arterial wall the lipids and LDL are subjected to oxidation and enzymatic modification. The resulting oxLDL is taken up by macrophages (V) via scavenger receptors and phagocytosis in uncontrolled fashion [84]. Recent studies have also highlighted the intra-plaque proliferation of macrophages in the lipid-rich stage of atherosclerotic plaque development (VI) [85]. Accumulation and uptake of oxLDL by macrophages triggers the secretion of chemotactic molecules [86], which promote the migration of SMC from the *media* into the subendothelial tissue. The intimal SMC lose their ability to contract, proliferate and synthesize extracellular matrix which results in fibrosis as part of the plaque development. The deposition of the extracellular matrix leads to further accumulation of oxLDL and lipids, in particular cholesterol and cholesteryl esters. The uptake of oxLDL via scavenger receptors is not subjected to negative feedback regulation, thus resulting in excessive intracellular lipid accumulation and formation of macrophage foam cells (VII) [87], as well as the release of chemotactic mediators. During further progression of atherosclerosis, fatty streaks are formed and the thickening of the vascular wall progresses through the ongoing deposition of extracellular lipids and proliferation of intimal SMC, and the accompanied synthesis of extracellular matrix proteins. Over many years these processes together form the characteristic necrotic lipid core covered and stabilized by a fibrotic cap (VIII). The stability of the atherosclerotic plaque is defined by the amount of accumulated lipids and also by the amount and quality of the extracellular matrix of the fibrotic cap; the progressing accumulation of lipids is often accompanied by reduced stability of the fibrotic cap (IX). Weakening of the cap may finally result in rupturing of the plaque, particularly in areas rich in macrophages as these cells produce a broad range of proteases that degrade the extracellular matrix of the fibrotic cap [88,89]. Plaque rupture causes thrombus formation (X) via the activation of the coagulation cascade [87]. Thrombi may occlude the artery at the site of plaque rupture or may flow through the blood stream and occlude downstream arteries that have a smaller lumen. In some cases, thrombi at the plaque site are reorganized and integrated into the plaque. This finally leads to the formation of the so-called complicated plaque (not shown).

isomers may have a variety of further independent properties, namely the modulation of gene expression, particularly that of genes encoding proteins involved in signaling but also the uptake, transport and degradation of tocopherols, as well as the uptake of lipoproteins and the storage and export of lipids such as cholesterol. The *in vitro* and *ex vivo* effects of α-tocopherol on cellular processes are depicted in Fig. 3.

α-Tocopherol metabolites and their bioactivity

Metabolic degradation of α-TOH takes place almost exclusively in the liver. Beside metabolites resulting from oxidation of the chroman moiety, hepatic metabolism of α-TOH involves CYP3A4[6]-dependent ω-hydroxylation and α-oxidation, which results in the formation of the *α*-tocopherol long-chain metabolites (α-LCM[7]) α-13′-OH[8] (13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanol) and α-13′-COOH[9] (13′-(6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-yl)-2,6,10-trimethyl-tridecanoic acid), and further steps of β-oxidation, which results in the formation of middle- and short-chain metabolites (α-SCM[10]) with the catabolic end-product α-carboxyethyl-hydroxychroman (α-CEHC[11]), respectively [8–10]. The short-chain metabolites are excreted via urine and are often used as a marker for α-TOH supply [11]. Other tocopherols, such as γ- and δ-tocopherol, are almost quantitatively degraded and excreted via the urine as the corresponding γ- and δ-CEHCs. The hepatic metabolism of α-TOH is illustrated in Fig. 4.

Regulatory activity is not restricted to α-TOH as its short-chain metabolite α-CEHC also exhibits bioactivity. It has been shown that α-CEHC is anti-proliferative [12], anti-inflammatory [13], and anti-oxidative [14], and inhibits oxLDL formation [15] and protein kinase C (PKC[12]) signaling [16]. Recently, researchers focused also on investigating the cellular effects of the α-LCM as α-13′-COOH was detected in human serum, a finding providing clear evidence for its systemic bioavailability. Until now, only a few cellular effects of the α-LCM have been described, such as pro-apoptotic, anti-proliferative and anti-inflammatory features [17–20], which are highlighted in Fig. 4.

[6] CYP3A4, cytochrome P450, subfamily IIIA, polypeptide 4.
[7] α-LCM, α-tocopherol long-chain metabolite(s).
[8] α-13′-OH, α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanol.
[9] α-13′-COOH, α-13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid.
[10] α-SCM, α-tocopherol short-chain metabolite(s).
[11] α-CEHC, α-carboxyethyl-hydroxychroman.
[12] PKC, protein kinase C.

Endothelial cells			
Signaling	⇩ PKC	⇩ NFκB activation	⇧ PPARγ DNA binding
Inflammatory process	⇩ Migration	⇩ Glucose-induced IL-8 release	⇧ Catalase expression
	⇩ Stimulus-induced VCAM-1, ICAM-1, E-selectin	⇩ H_2O_2-induced lipid peroxidation	⇩ Intracellular ROS production
	⇩ Monocyte adhesion to ECs	⇧ H_2O_2 degradation activity	⇩ Apoptosis by scavenging ROS
	⇩ Platelet adhesion on ECs	⇧ Intracellular GSH level	⇩ Apoptosis by inhibiting caspase activity
	⇧ eNOS activity		
Monocytes and macrophages			
Lipid homeostasis	⇩ CD36	⇩ ABCA1/ABCG1	
	⇩ SR-A	⇩ oxLDL-induced ABCA1/G1 expression	
Signaling	⇩ NFκB activation		
Inflammatory process	⇩ Migration	⇩ Stimulus-induced COX-2 and iNOS expression as well as PGE_2 and NO release	⇩ Stimulus-induced release of IL-1β, IL-6, TNF-α, IFN-γ, MCP-1 and IL-8
	⇩ Stimulus-induced adhesion		
	⇩ Stimulus-induced CD11b, VCAM-1, VLA-4	⇩ 7-Ketocholesterol induced apoptosis	⇩ Superoxide anion release
Smooth muscle cells			
Lipid homeostasis	⇩ CD36	⇩ oxLDL uptake	
Signaling	⇩ PKC	⇩ PMA-induced ERK activation	⇩ Akt-PKB dephosphorylation
	⇧ Protein phosphatase 2A	⇧ DAG kinase activation	
Inflammatory process	⇩ Migration	⇩ SMC proliferation by PKC inhibition	⇩ 7-Ketocholesterol induced apoptosis
Plaque stability	⇧ CTGF (connective tissue growth factor, stimulates synthesis of extracellular matrix) expression		
Hepatocytes			
Lipid homeostasis	⇩ CD36	⇩ Isopentenyl-diphosphate δ isomerase	⇩ Squalene synthase
	⇩ LDL-R	⇩ Farnesyl diphosphate synthase	⇧ 27-Hydroxycholesterol under high C-diet
	⇩ HMG-CoA reductase and synthase	⇩ 7-Dehydrocholesterol reductase	⇧ CYP27A1 under high C-diet
Signaling	⇩ NFκB activation		
Inflammatory process	⇩ Stimulus enhanced iNOS expression		
xenobiotic metabolism	⇧ CYP3A11 to levels which might interfere with drug metabolism		
Non-cellular			
Plaque stability	⇧ Increased number of plaques with thicker cap	⇩ Percentage of ruptured plaques	⇩ oxLDL formation
	⇩ Necrotic core areas	⇩ MMP3 expression	⇩ Long-term
			⇩/⇧ Short- term: depending on condition

Fig. 3. Atherosclerosis-relevant *in vitro* and *ex vivo* effects of α-tocopherol. Due to its function as an antioxidant, vitamin E was considered to interfere with key events in atherogenesis. To gain better insights into the contribution of vitamin E to the molecular processes underlying the hallmarks of atherosclerosis much effort was spend on *in vitro* and *ex vivo* experiments as well as studies involving animal models. As the complex pathogenesis of atherosclerosis involves several different cell types, the figure is divided according to the cells of interest (EC, SMC, monocytes and macrophages, hepatocytes) as well as non-cellular, plaque-specific processes and categories such as lipid homeostasis, signaling, inflammation, plaque stability and xenobiotic metabolism which are used to reflect the hallmarks of atherosclerosis. Endothelial cells surface the arterial wall and their dysfunction is the initial step of atherogenesis. α-Tocopherol reduces the stimulus-induced expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and E-selectin [90–92], and decreases thereby the adhesion [92] and immigration of leukocytes [93] onto the endothelium or into the arterial wall, respectively. α-Tocopherol makes it easier for EC to deal with oxidative stress, such as H_2O_2-induced lipid peroxidation [7], due to higher catalase expression [94], increased H_2O_2 degradation activity, and higher intracellular GSH levels [13]. Thus, apoptosis of EC induced by oxidative stress is reduced [95]. Beside reducing the release of inflammatory cytokines, such as interleukin (IL) 8 [96], in response to external stimuli, α-TOH interferes with cellular signaling by inhibiting, for example, PKC [97], activate nuclear factor κB (NFκB) [98] or binding of peroxisome proliferator-activated receptor γ (PPARγ) to its regulatory DNA elements [94]. Lipid homeostasis of SMC is also affected by α-TOH, for example by down-regulation of the scavenger receptor CD36 leading to reduced uptake of oxLDL [99]. α-Tocopherol inhibits migration [100] and proliferation of SMC by inhibiting PKC [101], and prevents 7-ketocholesterol-induced apoptosis [102]. Plaque stability is modulated by connective tissue growth factor (CTGF), which stimulates the synthesis of extracellular matrix. This factor is induced by α-TOH in SMC [103], suggesting that α-TOH contributes to plaque stability by inducing fibrotic processes. Several signaling cascades in SMC are also modulated by α-TOH, namely PKC [104,105], extracellular signal-regulated kinase (ERK) [106], protein kinase B (PK/AKT) and protein phosphatase 2A [107]. Monocytes migrate in the arterial wall, differentiate into macrophages and transform to foam cells under atherogenic conditions. α-Tocopherol reduces adhesion and migration of leukocytes by down-regulating expression of CD11b [93] and very late antigen-4 (VLA-4) [92,108]. Macrophage foam cell formation is prevented by reducing the expression of scavenger receptors CD36 [109,110] and A [111]. On the other hand, foam cell formation is triggered by the down-regulation of the lipid exporters ATP-binding cassette transporter (ABC) A1 and G1 [110]. Furthermore, the inflammatory response of macrophages to stimuli such as lipopolysaccharide (LPS) is dampened. The diminished induction of COX2 and inducible nitric oxide synthase (iNOS) by α-TOH results in a reduced release of prostaglandin E_2 (PGE_2) and nitric oxide [112,113]. The release of pro-inflammatory cytokines such as IL-1b, IL-6, tumor necrosis factor α (TNFα), and interferon γ (IFNγ) is also reduced [113]. Similar to SMC, 7-ketocholesterol-induced apoptosis is reduced by α-TOH in macrophages [114,115]. Signaling pathways, such as NFκB [108] and oxLDL or lipid-free high density lipoprotein induced liver X receptor α (LXRα) activity, are also inhibited by α-TOH [110]. The liver is the major organ for cholesterol biosynthesis and metabolism of lipoproteins, xenobiotics and α-TOH. In liver cells α-TOH down-regulates expression of CD36 [116,117] and the LDL receptor [118], as well as expression of enzymes involved in cholesterol biosynthesis, such as HMG-CoA reductase and HMG-CoA synthase [118]. In contrast, expression of cytochrome P450 subfamily 27A polypeptide 1 (CYP27A1), and thus synthesis of 27-hydroxycholesterol, is induced by α-TOH [119]. Similar to the situation in macrophages, activation of NFκB and expression of iNOS in response to stimuli is reduced by α-TOH [120]. α-Tocopherol also induces CYP3A11 to levels which might interfere with drug metabolism [121]. In addition to the effects of α-TOH on the different cell types involved in atherogenesis, α-TOH improves plaque stability in hyperlipidemic rabbits. Treatment of the animals with α-TOH increased the number of plaques with thicker, stabilizing fibrotic caps and reduced necrotic lipid core areas as well as reduced number of ruptured plaques [122]. Formation of oxLDL was also blocked by α-TOH [123]. Several studies using animals such as hypercholesterolemic rabbits or mice suggest that vitamin E inhibits atherogenesis in early [124] or advanced stages by its antioxidant capacity [125] or gene regulatory potential via signal transduction cascades and on adhesion molecules [126,127]. While high dose supplementation of vitamin E can improve myocardial tolerance to ischemia and reperfusion [128], Keaney et al. found that low dose α-TOH improves and high dose worsens endothelial vasodilatory function in cholesterol-fed rabbits [129]. Under extreme conditions, such as pronounced elevation in systemic oxidative stress due to hyperlipidaemia and obesity, vitamin E seems to be not cardioprotective [130].

A recent study by our group showed that α-LCM also affect macrophage foam cell formation by regulating uptake of oxLDL by macrophages via down-regulation of its phagocytic uptake (Fig. 4) [17]. A key finding of our study was that bioactivity of the α-LCMs occurs at much lower concentrations and with mechanisms distinct from those of their metabolic precursor α-TOH.

Perspective

The findings obtained from clinical trials with humans raise the question whether vitamin E *in vivo* exhibits modes of action different from those found *in vitro*. Recent studies shed new light on mechanistic aspects of α-TOH function, which appear to be complicated by

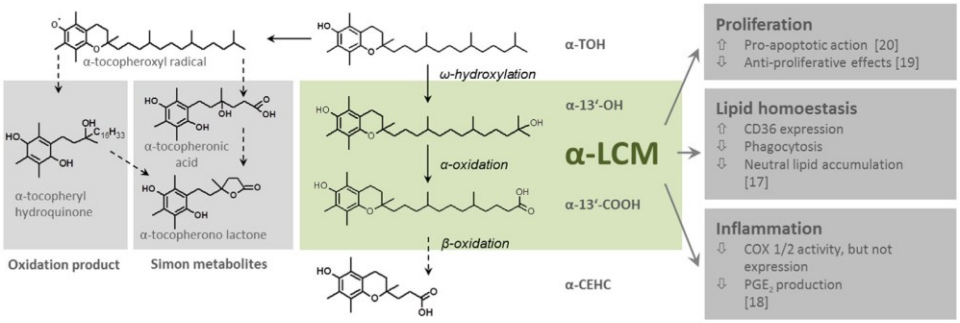

Fig. 4. α-Tocopherol metabolism and *in vitro* characteristics of its liver-derived long-chain metabolites. Due to the antioxidant capacity of vitamin E, early studies on its metabolism have concentrated on metabolites resulting from oxidation of the chroman moiety. The major oxidation product in the liver was described as α-tocopheryl quinone. This metabolite derives from the reaction of the tocopheroxyl radical with a peroxyl radical; it can be reduced to α-tocopheryl hydroquinone by NAD(P)H-dependent microsomal and mitochondrial enzymes. For many years, the so-called Simon metabolites, α-tocopheronic acid and its lactone, were the only known urinary α-TOH metabolites. The Simon metabolites are characterized by the opened chroman ring. Opening of the chroman ring starts with the formation of an α-tocopheroxyl radical when α-TOH has exerted its antioxidant activity. The Simon metabolites were therefore considered as urinary indicators that α-TOH had reacted as an antioxidant [131]. Today some researcher rise the question whether Simon metabolites are artefacts produced during sample preparation as α-CEHC is easily converted to α-tocopheronolactone by oxygenation [10,132]. α-Tocopherol is physiologically catabolized in the liver via the xenobiotic detoxification system involving CYP3A4 [8] and CYP4F2[133]. Cytochrome-dependent *ω*-hydroxylation results in the formation of the long-chain alcohol derivate α-13′-OH, 13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanol. Subsequent α-oxidation leads to α-13′-COOH, 13′-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid. The following β-oxidation steps in peroxisomes and mitochondria form the α-SCM, α-carboxyethyl-hydroxychroman (α-CEHC) [6]. This end-product of α-TOH metabolism can be conjugated and is excreted via urine [134]. The intact chroman structure indicates that α-CEHC is derived from α-TOH that has not reacted as an antioxidant. As α-CEHC excretion increases when certain plasma levels of *RRR*-α-TOH are exceeded, excretion of α-CEHC is considered as an indicator of adequate or excessive α-TOH supply. Although hepatic metabolism of α-TOH and the formation of the metabolic long- and short-chain intermediates are known for several years [8–10], the physiological function of the α-LCM α-13′-OH and α-13′-COOH is still unknown. Due to a lack of the pure compounds α-13′-OH and α-13′-COOH, only a few studies on the function of these α-LCM have been performed. Work so far focused on anti-proliferative effects, modulation of inflammatory processes and modulation of lipid homeostasis. Our group described anti-proliferative effects of the α-LCM due to pro-apoptotic action [20]. In HepG2 cells, the α-LCM induced cleavage of caspases 3, 7 and 9 as well as PARP-1 and induced mitochondrial dysfunction as characterized by reduced mitochondrial membrane potential and induced intra-mitochondrial ROS formation. The anti-proliferative effect of α-13′-COOH was shown also in murine glioma C6 cancer cells [19]. Others have reported that the α-LCM interfere with inflammatory processes by modulating activity of COX1 and COX2 and consequently by blocking production of PGE_2 [18]. Recent work by our group focused on the effects of the α-LCM on macrophage foam cell formation [17]. We have shown that α-LCM induce expression of the scavenger receptor CD36, the major receptor responsible for oxLDL uptake, in human macrophages, in contrast to the inhibiting actions of α-TOH [17]. Despite up-regulation of CD36, uptake of oxLDL and oxLDL-induced lipid accumulation was reduced in human macrophages, similar to the effects of α-TOH on oxLDL-mediated foam cell formation. An important finding of this recent study was that the metabolite α-13′-COOH was detected in serum providing for the first time evidence for the bioavailability of the α-LCM outside the liver. Another key finding of the study was that bioactivity of the α-LCM occur at lower concentrations and with mechanisms distinct from those of α-TOH. Taken together, these recent studies provide evidence for a role of the α-LCM as signaling molecules derived metabolically from α-TOH.

α-LCM circulating in the blood. We speculate that the α-LCM represent a new class of regulatory metabolites and propose that unraveling the molecular modes of action of the α-LCM and identifying the key players involved in their signaling may provide new fundamental insights into the biology and mode of function of vitamin E. Further studies are therefore required to elucidate the physiological role of the α-LCM and their contribution to disease processes, such as atherosclerosis. We also hypothesize that the discrepancy between the results obtained *in vitro* and *in vivo* in humans may be due to the physiologic metabolism of α-TOH and the formation of α-LCM in the liver and their release into circulation.

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Disclosures

None.

References

[1] P. Cullen, J. Rauterberg, S. Lorkowski, The pathogenesis of atherosclerosis, Handb. Exp. Pharmacol. 170 (2005) 3–70.
[2] H. Monajemi, E.K. Arkenbout, H. Pannekoek, Gene expression in atherogenesis, Thromb. Haemost. 86 (2001) 404–412.
[3] H. Jakubowski, The pathophysiological hypothesis of homocysteine thiolactone-mediated vascular disease, J. Physiol. Pharmacol. 59 (Suppl 9) (2008) 155–167.
[4] F.J. Raal, Pathogenesis and management of the dyslipidemia of the metabolic syndrome, Metab. Syndr. Relat. Disord. 7 (2009) 83–88.
[5] I.J. Kullo, G.T. Gau, A.J. Tajik, Novel risk factors for atherosclerosis, Mayo Clin. Proc. 75 (2000) 369–380.
[6] D.J. Mustacich, S.W. Leonard, N.K. Patel, M.G. Traber, Alpha-tocopherol beta-oxidation localized to rat liver mitochondria, Free Radic. Biol. Med. 48 (2010) 73–81.
[7] B. van Dam, van Hinsbergh, W.M. Victor, Stehouwer, D.A. Coen, A. Versteilen, H. Dekker, R. Buytenhek, H.M. Princen, C.G. Schalkwijk, Vitamin E inhibits lipid peroxidation-induced adhesion molecule expression in endothelial cells and decreases soluble cell adhesion molecules in healthy subjects, Cardiovasc. Res., 57, 563–571.
[8] M. Birringer, D. Drogan, R. Brigelius-Flohé, Tocopherols are metabolized in HepG2 cells by side chain omega-oxidation and consecutive beta-oxidation, Free Radic. Biol. Med. 31 (2001) 226–232.
[9] Y. Zhao, M.-J. Lee, C. Cheung, J.-H. Ju, Y.-K. Chen, B. Liu, L.-Q. Hu, C.S. Yang, Analysis of multiple metabolites of tocopherols and tocotrienols in mice and humans, J. Agric. Food Chem. 58 (2010) 4844–4852.
[10] M. Schultz, M. Leist, M. Petrzika, B. Gassmann, R. Brigelius-Flohé, Novel urinary metabolite of alpha-tocopherol, 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman, as an indicator of an adequate vitamin E supply? Am. J. Clin. Nutr. 62 (1995) 1527S–1534S.
[11] K.M. Lebold, A. Ang, M.G. Traber, L. Arab, Urinary α-carboxyethyl hydroxychroman can be used as a predictor of α-tocopherol adequacy, as demonstrated in the energetics study, Am. J. Clin. Nutr. 96 (2012) 801–809.
[12] F. Galli, A.M. Stabile, M. Betti, C. Conte, A. Pistilli, M. Rende, A. Floridi, A. Azzi, The effect of α- and γ-tocopherol and their carboxyethyl hydroxychroman metabolites on prostate cancer cell proliferation, Arch. Biochem. Biophys. 423 (2004) 97–102.
[13] P. Grammas, L. Hamdheydari, E.J. Benaksas, S. Mou, Q.N. Pye, W.J. Wechter, R.A. Floyd, C. Stewart, K. Hensley, Anti-inflammatory effects of tocopherol metabolites, Biochem. Biophys. Res. Commun. 319 (2004) 1047–1052.

[14] A. Betancor-Fernandez, H. Sies, W. Stahl, M.C. Polidori, In vitro antioxidant activity of 2,5,7,8-tetramethyl-2-(2′-carboxyethyl)-6-hydroxychroman (alpha-CEHC), a vitamin E metabolite, Free Radic. Res. 36 (2002) 915–921.
[15] H. Uto-Kondo, C. Kiyose, R. Ohmori, H. Saito, C. Taguchi, Y. Kishimoto, N. Machida, M. Hasegawa, E. Yoshioka, E. Saita, Y. Hirata, O. Igarashi, K. Kondo, The coantioxidative effects of carboxyethyl-6-hydroxychromans and alpha-Tocopherol, J. Nutr. Sci. Vitaminol. 53 (2007) 301–305.
[16] Z. Varga, E. Kosaras, E. Komodi, M. Katko, I. Karpati, J. Balla, G. Paragh, M.C. Aisa, F. Galli, Effects of tocopherols and 2,2′-carboxyethyl hydroxychromans on phorbol-ester-stimulated neutrophils, J. Nutr. Biochem. 19 (2008) 320–327.
[17] M. Wallert, S. Mosig, K. Rennert, H. Funke, M. Ristow, R.M. Pellegrino, G. Cruciani, F. Galli, S. Lorkowski, M. Birringer, Long-chain metabolites of α-tocopherol occur in human serum and inhibit macrophage foam cell formation in vitro, Free Radic. Biol. Med. 68 (2013) 43–51.
[18] Q. Jiang, X. Yin, M.A. Lill, M.L. Danielson, H. Freiser, J. Huang, Long-chain carboxychromanols, metabolites of vitamin E, are potent inhibitors of cyclooxygenases, Proc. Natl. Acad. Sci. USA 105 (2008) 20464–20469.
[19] F. Mazzini, M. Betti, T. Netscher, F. Galli, P. Salvadori, Configuration of the vitamin E analogue garcinoic acid extracted from garcinia kola seeds, Chirality 21 (2009) 519–524.
[20] M. Birringer, D. Lington, S. Vertuani, S. Manfredini, D. Scharlau, M. Glei, M. Ristow, Proapoptotic effects of long-chain vitamin E metabolites in HepG2 cells are mediated by oxidative stress, Free Radic. Biol. Med. 49 (2010) 1315–1322.
[21] R. Rodrigo, C. Guichard, R. Charles, Clinical pharmacology and therapeutic use of antioxidant vitamins, Fundam. Clin. Pharmacol. 21 (2007) 111–127.
[22] M.J. Stampfer, C.H. Hennekens, J.E. Manson, G.A. Colditz, B. Rosner, W.C. Willett, Vitamin E consumption and the risk of coronary disease in women, N. Engl. J. Med. 328 (1993) 1444–1449.
[23] S. Yusuf, G. Dagenais, J. Pogue, J. Bosch, P. Sleight, Vitamin E supplementation and cardiovascular events in high-risk patients. The heart outcomes prevention evaluation study investigators, N. Engl. J. Med. 342 (2000) 154–160.
[24] S. Hercberg, P. Galan, P. Preziosi, S. Bertrais, L. Mennen, D. Malvy, A.-M. Roussel, A. Favier, S. Briançon, The SU.VI.MAX study: a randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals, Arch. Intern. Med. 164 (2004) 2335–2342.
[25] Gruppo Italiano per lo Studio della, Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Sopravvivenza nell'Infarto miocardico, Lancet 354, 1999, pp. 447–455.
[26] M.E. Wright, K.A. Lawson, S.J. Weinstein, P. Pietinen, P.R. Taylor, J. Virtamo, D. Albanes, Higher baseline serum concentrations of vitamin E are associated with lower total and cause-specific mortality in the alpha-tocopherol, beta-carotene cancer prevention study, Am. J. Clin. Nutr. 84 (2006) 1200–1207.
[27] J.M. Rapola, J. Virtamo, S. Ripatti, J.K. Huttunen, D. Albanes, P.R. Taylor, O.P. Heinonen, Randomised trial of alpha-tocopherol and beta-carotene supplements on incidence of major coronary events in men with previous myocardial infarction, Lancet 349 (1997) 1715–1720.
[28] J. Virtamo, J.M. Rapola, S. Ripatti, O.P. Heinonen, P.R. Taylor, D. Albanes, J.K. Huttunen, Effect of vitamin E and beta carotene on the incidence of primary nonfatal myocardial infarction and fatal coronary heart disease, Arch. Intern. Med. 158 (1998) 668–675.
[29] J.M. Leppälä, J. Virtamo, R. Fogelholm, J.K. Huttunen, D. Albanes, P.R. Taylor, O.P. Heinonen, Controlled trial of alpha-tocopherol and beta-carotene supplements on stroke incidence and mortality in male smokers, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 230–235.
[30] T. Hirvonen, J. Virtamo, P. Korhonen, D. Albanes, P. Pietinen, Intake of flavonoids, carotenoids, vitamins C and E, and risk of stroke in male smokers, Stroke 31 (2000) 2301–2306.
[31] J. Muntwyler, C.H. Hennekens, J.E. Manson, J.E. Buring, J.M. Gaziano, Vitamin supplement use in a low-risk population of US male physicians and subsequent cardiovascular mortality, Arch. Intern. Med. 162 (2002) 1472–1476.
[32] E.B. Rimm, M.J. Stampfer, A. Ascherio, E. Giovannucci, G.A. Colditz, W.C. Willett, Vitamin E consumption and the risk of coronary heart disease in men, N. Engl. J. Med. 328 (1993) 1450–1456.
[33] A. Ascherio, E.B. Rimm, M.A. Hernán, E. Giovannucci, I. Kawachi, M.J. Stampfer, W.C. Willett, Relation of consumption of vitamin E, vitamin C, and carotenoids to risk for stroke among men in the United States, Ann. Intern. Med. 130 (1999) 963–970.
[34] B. Frei, J.M. Gaziano, Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation, J. Lipid Res. 34 (1993) 2135–2145.
[35] D.P. Vivekananthan, M.S. Penn, S.K. Sapp, A. Hsu, E.J. Topol, Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials, Lancet 361 (2003) 2017–2023.
[36] M. Meydani, P. Kwan, M. Band, A. Knight, W. Guo, J. Goutis, J. Ordovas, Long-term vitamin E supplementation reduces atherosclerosis and mortality in Ldlr−/− mice, but not when fed Western style diet, Atherosclerosis 233 (2014) 196–205.
[37] Z. Cordero, D. Drogan, C. Weikert, H. Boeing, Vitamin E and risk of cardiovascular diseases: a review of epidemiologic and clinical trial studies, Crit. Rev. Food Sci. Nutr. 50 (2010) 420–440.
[38] K. Asplund, Antioxidant vitamins in the prevention of cardiovascular disease: a systematic review, J. Intern. Med. 251 (2002) 372–392.
[39] R. Marchioli, Antioxidant vitamins and prevention of cardiovascular disease: laboratory, epidemiological and clinical trial data, Pharmacol. Res. 40 (1999) 227–238.
[40] P. Knekt, J. Ritz, M.A. Pereira, E.J. O'Reilly, K. Augustsson, G.E. Fraser, U. Goldbourt, B.L. Heitmann, G. Hallmans, S. Liu, P. Pietinen, D. Spiegelman, J. Stevens, J. Virtamo, W.C. Willett, E.B. Rimm, A. Ascherio, Antioxidant vitamins and coronary heart disease risk: a pooled analysis of 9 cohorts, Am. J. Clin. Nutr. 80 (2004) 1508–1520.
[41] R.S. Eidelman, D. Hollar, P.R. Hebert, G.A. Lamas, C.H. Hennekens, Randomized trials of vitamin E in the treatment and prevention of cardiovascular disease, Arch. Intern. Med. 164 (2004) 1552–1556.
[42] P.G. Shekelle, S.C. Morton, L.K. Jungvig, J. Udani, M. Spar, W. Tu, M.J. Suttorp, I. Coulter, S.J. Newberry, M. Hardy, Effect of supplemental vitamin E for the prevention and treatment of cardiovascular disease, J. Gen. Intern. Med. 19 (2004) 380–389.
[43] M.R. Law, J.K. Morris, By how much does fruit and vegetable consumption reduce the risk of ischaemic heart disease? Eur. J. Clin. Nutr. 52 (1998) 549–556.
[44] E.R. Miller, R. Pastor-Barriuso, D. Dalal, R.A. Riemersma, L.J. Appel, E. Guallar, Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality, Ann. Intern. Med. 142 (2005) 37–46.
[45] J.T. Salonen, K. Nyyssönen, R. Salonen, H.M. Lakka, J. Kaikkonen, E. Porkkala-Sarataho, S. Voutilainen, T.A. Lakka, T. Rissanen, L. Leskinen, T.P. Tuomainen, V.P. Valkonen, U. Ristonmaa, H.E. Poulsen, Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study: a randomized trial of the effect of vitamins E and C on 3-year progression of carotid atherosclerosis, J. Intern. Med. 248 (2000) 377–386.
[46] K. Kostner, S. Hornykewycz, P. Yang, T. Neunteufl, D. Glogar, F. Weidinger, G. Maurer, K. Huber, Is oxidative stress causally linked to unstable angina pectoris? A study in 100 CAD patients and matched controls, Cardiovasc. Res. 36 (1997) 330–336.
[47] N.G. Stephens, A. Parsons, P.M. Schofield, F. Kelly, K. Cheeseman, M.J. Mitchinson, Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS), Lancet 347 (1996) 781–786.
[48] M.L. Watkins, J.D. Erickson, M.J. Thun, J. Mulinare, C.W. Heath, Multivitamin use and mortality in a large prospective study, Am. J. Epidemiol. 152 (2000) 149–162.
[49] A. El-Sohemy, A. Baylin, D. Spiegelman, A. Ascherio, H. Campos, Dietary and adipose tissue gamma-tocopherol and risk of myocardial infarction, Epidemiology 13 (2002) 216–223.
[50] M. Bobák, E. Brunner, N.J. Miller, Z. Skodová, M. Marmot, Could antioxidants play a role in high rates of coronary heart disease in the Czech Republic? Eur. J. Clin. Nutr. 52 (1998) 632–636.
[51] J.T. Salonen, R. Salonen, I. Penttilä, J. Herranen, M. Jauhiainen, M. Kantola, R. Lappeteläinen, P.H. Mäenpää, G. Alfthan, P. Puska, Serum fatty acids, apolipoproteins, selenium and vitamin antioxidants and the risk of death from coronary artery disease, Am. J. Cardiol. 56 (1985) 226–231.
[52] F.J. Kok, A.M. de Bruijn, R. Vermeeren, A. Hofman, A. van Laar, M. de Bruin, R.J. Hermus, H.A. Valkenburg, Serum selenium, vitamin antioxidants, and cardiovascular mortality: a 9-year follow-up study in the Netherlands, Am. J. Clin. Nutr. 45 (1987) 462–468.
[53] K.G. Losonczy, T.B. Harris, R.J. Havlik, Vitamin E and vitamin C supplement use and risk of all-cause and coronary heart disease mortality in older persons: the established populations for epidemiologic studies of the elderly, Am. J. Clin. Nutr. 64 (1996) 190–196.
[54] P. Knekt, A. Reunanen, R. Järvinen, R. Seppänen, M. Heliövaara, A. Aromaa, Antioxidant vitamin intake and coronary mortality in a longitudinal population study, Am. J. Epidemiol. 139 (1994) 1180–1189.
[55] B.G. Brown, X.Q. Zhao, A. Chait, L.D. Fisher, M.C. Cheung, J.S. Morse, A.A. Dowdy, E.K. Marino, E.L. Bolson, P. Alaupovic, J. Frohlich, J.J. Albers, Simvastatin and niacin, antioxidant vitamins, or the combination for the prevention of coronary disease, N. Engl. J. Med. 345 (2001) 1583–1592.
[56] Y. Ito, K. Suzuki, J. Ishii, H. Hishida, A. Tamakoshi, N. Hamajima, K. Aoki, A population-based follow-up study on mortality from cancer or cardiovascular disease and serum carotenoids, retinol and tocopherols in Japanese inhabitants, Asian Pac. J. Cancer Prev. 7 (2006) 533–546.
[57] E. Lonn, J. Bosch, S. Yusuf, P. Sheridan, J. Pogue, J. Arnold, O. Malcolm, C. Ross, A. Arnold, P. Sleight, J. Probstfield, G.R. Dagenais, Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial, JAMA 293 (2005) 1338–1347.
[58] L.H. Kushi, A.R. Folsom, R.J. Prineas, P.J. Mink, Y. Wu, R.M. Bostick, Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women, N. Engl. J. Med. 334 (1996) 1156–1162.
[59] S. Nojiri, H. Daida, H. Mokuno, Y. Iwama, K. Mae, F. Ushio, T. Ueki, Association of serum antioxidant capacity with coronary artery disease in middle-aged men, Jpn. Heart J. 42 (2001) 677–690.
[60] S.D. Mark, W. Wang, J.F. Fraumeni, J.Y. Li, P.R. Taylor, G.Q. Wang, W. Guo, S.M. Dawsey, B. Li, W.J. Blot, Lowered risks of hypertension and cerebrovascular disease after vitamin/mineral supplementation: the Linxian Nutrition Intervention Trial, Am. J. Epidemiol. 143 (1996) 658–664.
[61] W.J. Blot, J.Y. Li, P.R. Taylor, W. Guo, S.M. Dawsey, B. Li, The Linxian trials: mortality rates by vitamin-mineral intervention group, Am. J. Clin. Nutr. 62 (1995) 1424S–1426S.

[62] Heart Protection Study Collaborative Group, MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20 536 high-risk individuals: a randomised placebo-controlled trial//MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high-risk individuals: a randomised placebo-controlled trial, Lancet 360 (2002) 23–33.

[63] H.W. Hense, M. Stender, W. Bors, U. Keil, Lack of an association between serum vitamin E and myocardial infarction in a population with high vitamin E levels, Atherosclerosis 103 (1993) 21–28.

[64] N.R. Sahyoun, P.F. Jacques, R.M. Russell, Carotenoids, vitamins C and E, and mortality in an elderly population, Am. J. Epidemiol. 144 (1996) 501–511.

[65] H.D. Sesso, J.E. Buring, W.G. Christen, T. Kurth, C. Belanger, J. MacFadyen, V. Bubes, J.E. Manson, R.J. Glynn, J.M. Gaziano, Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial, JAMA 300 (2008) 2123–2133.

[66] K. Klipstein-Grobusch, J.M. Geleijnse, J.H. den Breeijen, H. Boeing, A. Hofman, D.E. Grobbee, J.C. Witteman, Dietary antioxidants and risk of myocardial infarction in the elderly: the Rotterdam Study, Am. J. Clin. Nutr. 69 (1999) 261–266.

[67] R.A. Riemersma, D.A. Wood, C.C. Macintyre, R.A. Elton, K.F. Gey, M.F. Oliver, Risk of angina pectoris and plasma concentrations of vitamins A, C, and E and carotene, Lancet 337 (1991) 1–5.

[68] S. Todd, M. Woodward, H. Tunstall-Pedoe, C. Bolton-Smith, Dietary antioxidant vitamins and fiber in the etiology of cardiovascular disease and all-causes mortality: results from the Scottish Heart Health Study, Am. J. Epidemiol. 150 (1999) 1073–1080.

[69] M. Boaz, S. Smetana, T. Weinstein, Z. Matas, U. Gafter, A. Iaina, A. Knecht, Y. Weissgarten, D. Brunner, M. Fainaru, M.S. Green, Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial, Lancet 356 (2000) 1213–1218.

[70] R.K. Ross, J.M. Yuan, B.E. Henderson, J. Park, Y.T. Gao, M.C. Yu, Prospective evaluation of dietary and other predictors of fatal stroke in Shanghai, China, Circulation 96 (1997) 50–55.

[71] R. Delport, J.B. Ubbink, J.A. Human, P.J. Becker, D.P. Myburgh, W.J. Vermaak, Antioxidant vitamins and coronary artery disease risk in South African males, Clin. Chim. Acta 278 (1998) 55–60.

[72] D. Rajasekhar, P.V.L.N. Srinivasa Rao, S.A.A. Latheef, K.S.S. Saibaba, G. Subramanyam, Association of serum antioxidants and risk of coronary heart disease in South Indian population, Indian J. Med. Sci. 58 (2004) 465–471.

[73] S. Meraji, P.M. Abuja, M. Hayn, G.M. Kostner, R. Morris, S. Oraii, F. Tatzber, W. Wonisch, R. Zechner, K.F. Gey, Relationship between classic risk factors, plasma antioxidants and indicators of oxidant stress in angina pectoris (AP) in Tehran, Atherosclerosis 150 (2000) 403–412.

[74] R.W. Evans, B.J. Shaten, B.W. Day, L.H. Kuller, Prospective association between lipid soluble antioxidants and coronary heart disease in men. The Multiple Risk Factor Intervention Trial, Am. J. Epidemiol. 147 (1998) 180–186.

[75] G. de Gaetano, Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. Collaborative Group of the Primary Prevention Project, Lancet 357 (2001) 89–95.

[76] S.O. Keli, M.G. Hertog, E.J. Feskens, D. Kromhout, Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study, Arch. Intern. Med. 156 (1996) 637–642.

[77] M. Feki, M. Souissi, E. Mokhtar, M. Hsairi, N. Kaabachi, H. Antebi, L.G. Alcindor, R. Mechmeche, A. Mebazaa, Vitamin E and coronary heart disease in Tunisians, Clin. Chem. 46 (2000) 1401–1405.

[78] G. Ferns, J. Williams, L. Forster, S. Tull, B. Starkey, A. Gershlick, Cholesterol standardized plasma vitamin E levels are reduced in patients with severe angina pectoris, Int. J. Exp. Pathol. 81 (2000) 57–62.

[79] N.R. Cook, C.M. Albert, J.M. Gaziano, E. Zaharris, J. MacFadyen, E. Danielson, J.E. Buring, J.E. Manson, A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: results from the Women's Antioxidant Cardiovascular Study, Arch. Intern. Med. 167 (2007) 1610–1618.

[80] I.-M. Lee, N.R. Cook, J.M. Gaziano, D. Gordon, P.M. Ridker, J.E. Manson, C.H. Hennekens, J.E. Buring, Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial, JAMA 294 (2005) 56–65.

[81] A.F. Kardinaal, F.J. Kok, J. Ringstad, J. Gomez-Aracena, V.P. Mazaev, L. Kohlmeier, B.C. Martin, A. Aro, J.D. Kark, M. Delgado-Rodriguez, Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC Study, Lancet 342 (1993) 1379–1384.

[82] J.L. Goldstein, Y.K. Ho, S.K. Basu, M.S. Brown, Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition, Proc. Natl. Acad. Sci. USA 76 (1979) 333–337.

[83] C.J. Schwartz, A.J. Valente, E.A. Sprague, J.L. Kelley, R.M. Nerem, The pathogenesis of atherosclerosis: an overview, Clin. Cardiol. 14 (1991) I1–16.

[84] L.-P. Erwig, P.M. Henson, Clearance of apoptotic cells by phagocytes, Cell Death Differ. 15 (2008) 243–250.

[85] C.S. Robbins, I. Hilgendorf, G.F. Weber, I. Theurl, Y. Iwamoto, J.-L. Figueiredo, R. Gorbatov, G.K. Sukhova, L.M. Gerhardt, M.S. Louisa, D. Smyth, Caleb C. J. Zavitz, E.A. Shikatani, M. Parsons, N. van Rooijen, H.Y. Lin, M. Husain, P. Libby, M. Nahrendorf, R. Weissleder, F.K. Swirski, Local proliferation dominates lesional macrophage accumulation in atherosclerosis, Nat. Med. 19 (2013) 1166–1172.

[86] S.D. Cushing, J.A. Berliner, A.J. Valente, M.C. Territo, M. Navab, F. Parhami, R. Gerrity, C.J. Schwartz, A.M. Fogelman, Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells, Proc. Natl. Acad. Sci. USA 87 (1990) 5134–5138.

[87] S. Collot-Teixeira, J. Martin, C. McDermott-Roe, R. Poston, J.L. McGregor, CD36 and macrophages in atherosclerosis, Cardiovasc. Res. 75 (2007) 468–477.

[88] A.C. Newby, Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 2108–2114.

[89] M. Bäck, D.F. Ketelhuth, S. Agewall, Matrix metalloproteinases in atherothrombosis, Prog. Cardiovas. Dis. 52 (2010) 410–428.

[90] U. Catalán, S. Fernández-Castillejo, L. Pons, M. Heras, G. Aragonés, N. Anglès, J.-R. Morelló, R. Solà, Alpha-tocopherol and BAY 11-7082 reduce vascular cell adhesion molecule in human aortic endothelial cells, J. Vasc. Res. 49 (2012) 319–328.

[91] D. Wu, T. Koga, K.R. Martin, M. Meydani, Effect of vitamin E on human aortic endothelial cell production of chemokines and adhesion to monocytes, Atherosclerosis 147 (1999) 297–307.

[92] N. Noguchi, R. Hanyu, A. Nonaka, Y. Okimoto, T. Kodama, Inhibition of THP-1 cell adhesion to endothelial cells by alpha-tocopherol and alpha-tocotrienol is dependent on intracellular concentration of the antioxidants, Free Radic. Biol. Med. 34 (2003) 1614–1620.

[93] S. Berdnikovs, H. Abdala-Valencia, C. McCary, M. Somand, R. Cole, A. Garcia, P. Bryce, J.M. Cook-Mills, Isoforms of vitamin E have opposing immunoregulatory functions during inflammation by regulating leukocyte recruitment, J. Immunol. 182 (2009) 4395–4405.

[94] Y.K. Nakamura, S.T. Omaye, Alpha-tocopherol modulates human umbilical vein endothelial cell expression of Cu/Zn superoxide dismutase and catalase and lipid peroxidation, Nutr. Res. 28 (2008) 671–680.

[95] M. Uemura, H. Manabe, N. Yoshida, N. Fujita, J. Ochiai, N. Matsumoto, T. Takagi, Y. Naito, T. Yoshikawa, Alpha-tocopherol prevents apoptosis of vascular endothelial cells via a mechanism exceeding that of mere antioxidation, Eur. J. Pharmacol. 456 (2002) 29–37.

[96] Y. Nobata, M. Urakaze, R. Temaru, A. Sato, N. Nakamura, K. Yamazaki, M. Kishida, M. Takata, M. Kobayashi, alpha-Tocopherol Inhibits IL-8 synthesis induced by thrombin and high glucose in endothelial cells, Horm. Metab. Res. 34 (2002) 49–54.

[97] C.A. McCary, Y. Yoon, C. Panagabko, W. Cho, J. Atkinson, J.M. Cook-Mills, Vitamin E isoforms directly bind PKCα and differentially regulate activation of PKCα, Biochem. J. 441 (2012) 189–198.

[98] D. Li, T. Saldeen, J.L. Mehta, Effects of alpha-tocopherol on ox-LDL-mediated degradation of IkappaB and apoptosis in cultured human coronary artery endothelial cells, J. Cardiovasc. Pharmacol. 36 (2000) 297–301.

[99] R. Ricciarelli, J.M. Zingg, A. Azzi, Vitamin E reduces the uptake of oxidized LDL by inhibiting CD36 scavenger receptor expression in cultured aortic smooth muscle cells, Circulation 102 (2000) 82–87.

[100] K. Yasunari, M. Kohno, H. Kano, K. Yokokawa, M. Minami, J. Yoshikawa, Antioxidants improve impaired insulin-mediated glucose uptake and prevent migration and proliferation of cultured rabbit coronary smooth muscle cells induced by high glucose, Circulation 99 (1999) 1370–1378.

[101] D. Boscoboinik, A. Szewczyk, A. Azzi, Alpha-tocopherol (vitamin E) regulates vascular smooth muscle cell proliferation and protein kinase C activity, Arch. Biochem. Biophys. 286 (1991) 264–269.

[102] M.-C. Royer, S. Lemaire-Ewing, C. Desrumaux, S. Monier, Pais de Barros, Jean-Paul, A. Athias, D. Néel, L. Lagrost, 7-ketocholesterol incorporation into sphingolipid/cholesterol-enriched (lipid raft) domains is impaired by vitamin E: a specific role for alpha-tocopherol with consequences on cell death, J. Biol. Chem. 284 (2009) 15826–15834.

[103] L. Villacorta, A.V. Graça-Souza, R. Ricciarelli, J.-M. Zingg, A. Azzi, Alpha-tocopherol induces expression of connective tissue growth factor and antagonizes tumor necrosis factor-alpha-mediated downregulation in human smooth muscle cells, Circ. Res. 92 (2003) 104–110.

[104] R. Ricciarelli, A. Tasinato, S. Clément, N.K. Ozer, D. Boscoboinik, A. Azzi, alpha-Tocopherol specifically inactivates cellular protein kinase C alpha by changing its phosphorylation state, Biochem. J. 334 (Pt 1) (1998) 243–249.

[105] A. Azzi, D. Boscoboinik, D. Marilley, N.K. Ozer, B. Stäuble, A. Tasinato, Vitamin E: a sensor and an information transducer of the cell oxidation state, Am. J. Clin. Nutr. 62 (1995) 1337S–1346S.

[106] S.A. Clément, C.C. Tan, J. Guo, K. Kitta, Y.J. Suzuki, Roles of protein kinase C and alpha-tocopherol in regulation of signal transduction for GATA-4 phosphorylation in HL-1 cardiac muscle cells, Free Radic. Biol. Med. 32 (2002) 341–349.

[107] A. Azzi, D. Boscoboinik, S. Clément, D. Marilley, N.K. Ozer, R. Ricciarelli, A. Tasinato, Alpha-tocopherol as a modulator of smooth muscle cell proliferation, Prostaglandins Leukot. Essent. Fatty Acids 57 (1997) 507–514.

[108] K.N. Islam, S. Devaraj, I. Jialal, alpha-Tocopherol enrichment of monocytes decreases agonist-induced adhesion to human endothelial cells, Circulation 98 (1998) 2255–2261.

[109] S. Devaraj, I. Hugou, I. Jialal, Alpha-tocopherol decreases CD36 expression in human monocyte-derived macrophages, J. Lipid Res. 42 (2001) 521–527.

[110] S. Rode, T. Rubic, R.L. Lorenz, alpha-Tocopherol disturbs macrophage LXRalpha regulation of ABCA1/G1 and cholesterol handling, Biochem. Biophys. Res. Commun. 369 (2008) 868–872.

[111] D. Teupser, J. Thiery, D. Seidel, Alpha-tocopherol down-regulates scavenger receptor activity in macrophages, Atherosclerosis 144 (1999) 109–115.

[112] S. Kim, E.-H. Lee, S.H. Kim, S. Lee, S.-J. Lim, Comparison of three tocopherol analogs as an inhibitor of production of proinflammatory mediators in macrophages, J. Pharmacol. Sci. 118 (2012) 237–244.
[113] L.-T. Ng, H.-J. Ko, Comparative effects of tocotrienol-rich fraction, α-tocopherol and α-tocopheryl acetate on inflammatory mediators and nuclear factor kappa B expression in mouse peritoneal macrophages, Food Chem. 134 (2012) 920–925.
[114] N.M. Lyons, J.A. Woods, N.M. O'Brien, alpha-Tocopherol, but not gamma-tocopherol inhibits 7 beta-hydroxycholesterol-induced apoptosis in human U937 cells, Free Radic. Res. 35 (2001) 329–339.
[115] A. Vejux, S. Guyot, T. Montange, J.-M. Riedinger, E. Kahn, G. Lizard, Phospholipidosis and down-regulation of the PI3-K/PDK-1/Akt signalling pathway are vitamin E inhibitable events associated with 7-ketocholesterol-induced apoptosis, J. Nutr. Biochem. 20 (2009) 45–61.
[116] G. Rimbach, A. Fischer, E. Stoecklin, L. Barella, Modulation of hepatic gene expression by alpha-tocopherol in cultured cells and in vivo, Ann. N. Y. Acad. Sci. 1031 (2004) 102–108.
[117] L. Barella, P.Y. Muller, M. Schlachter, W. Hunziker, E. Stöcklin, V. Spitzer, N. Meier, S. de Pascual-Teresa, A.-M. Minihane, G. Rimbach, Identification of hepatic molecular mechanisms of action of alpha-tocopherol using global gene expression profile analysis in rats, Biochim. Biophys. Acta 2004 (1689) 66–74.
[118] S. Valastyan, V. Thakur, A. Johnson, K. Kumar, D. Manor, Novel transcriptional activities of vitamin E: inhibition of cholesterol biosynthesis, Biochemistry 47 (2008) 744–752.
[119] J. Poirier, K.A. Cockell, W.M. NimalRatnayake, K.A. Scoggan, N. Hidiroglou, C. Gagnon, H. Rocheleau, H. Gruber, P. Griffin, R. Madère, K. Trick, S. Kubow, Antioxidant supplements improve profiles of hepatic oxysterols and plasma lipids in butter-fed hamsters, Nutr. Metab. Insights 3 (2010) 1–14.
[120] R. González, J.A. Collado, S. Nell, J. Briceño, M.J. Tamayo, E. Fraga, A. Bernardos, P. López-Cillero, J.M. Pascussi, S. Rufián, M.-J. Vilarem, ManuelDe la Mata, R. Brigelius-Flohe, P. Maurel, J. Muntané, Cytoprotective properties of alpha-tocopherol are related to gene regulation in cultured D-galactosamine-treated human hepatocytes, Free Radic. Biol. Med. 43 (2007) 1439–1452.
[121] D. Kluth, N. Landes, P. Pfluger, K. Müller-Schmehl, K. Weiss, C. Bumke-Vogt, M. Ristow, R. Brigelius-Flohé, Modulation of Cyp3a11 mRNA expression by alpha-tocopherol but not gamma-tocotrienol in mice, Free Radic. Biol. Med. 38 (2005) 507–514.
[122] W. Li, A. Hellsten, L.S. Jacobsson, H.M. Blomqvist, A.G. Olsson, X.-M. Yuan, Alpha-tocopherol and astaxanthin decrease macrophage infiltration, apoptosis and vulnerability in atheroma of hyperlipidaemic rabbits, J. Mol. Cell. Cardiol. 37 (2004) 969–978.
[123] L. Satchell, D.S. Leake, Oxidation of low-density lipoprotein by iron at lysosomal pH: implications for atherosclerosis, Biochemistry 51 (2012) 3767–3775.
[124] R.J. Williams, J.M. Motteram, C.H. Sharp, P.J. Gallagher, Dietary vitamin E and the attenuation of early lesion development in modified Watanabe rabbits, Atherosclerosis 94 (1992) 153–159.
[125] D. Praticò, R.K. Tangirala, D.J. Rader, J. Rokach, G.A. FitzGerald, Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in ApoE-deficient mice, Nat. Med. 4 (1998) 1189–1192.
[126] N.K. Ozer, O. Sirikçi, S. Taha, T. San, U. Moser, A. Azzi, Effect of vitamin E and probucol on dietary cholesterol-induced atherosclerosis in rabbits, Free Radic. Biol. Med. 24 (1998) 226–233.
[127] T. Koga, P. Kwan, L. Zubik, C. Ameho, D. Smith, M. Meydani, Vitamin E supplementation suppresses macrophage accumulation and endothelial cell expression of adhesion molecules in the aorta of hypercholesterolemic rabbits, Atherosclerosis 176 (2004) 265–272.
[128] R.A. Axford-Gately, G.J. Wilson, Myocardial infarct size reduction by single high dose or repeated low dose vitamin E supplementation in rabbits, Can. J. Cardiol. 9 (1993) 94–98.
[129] J.F. Keaney, J.M. Gaziano, A. Xu, B. Frei, J. Curran-Celentano, G.T. Shwaery, J. Loscalzo, J.A. Vita, Low-dose alpha-tocopherol improves and high-dose alpha-tocopherol worsens endothelial vasodilator function in cholesterol-fed rabbits, J. Clin. Invest. 93 (1994) 844–851.
[130] A.H. Hasty, M.L. Gruen, E.S. Terry, B.K. Surmi, R.D. Atkinson, L. Gao, J. D. Morrow, Effects of vitamin E on oxidative stress and atherosclerosis in an obese hyperlipidemic mouse model, J. Nutr. Biochem. 18 (2007) 127–133.
[131] R. Brigelius-Flohé, M.G. Traber, Vitamin E: function and metabolism, FEBS J. 13 (1999) 1145–1155.
[132] R. Brigelius-Flohé, F.J. Kelly, J.T. Salonen, J. Neuzil, J.-M. Zingg, A. Azzi, The European perspective on vitamin E: current knowledge and future research, Am. J. Clin. Nutr. 76 (2002) 703–716.
[133] T.J. Sontag, R.S. Parker, Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status, J. Biol. Chem. 277 (2002) 25290–25296.
[134] M.G. Traber, Mechanisms for the prevention of vitamin E excess, J. Lipid Res. 54 (2013) 2295–2306.

3.3 Manuscript III

The α-tocopherol long-chain metabolite α-13'-COOH affects the inflammatory burst of lipopolysaccharide-activated murine RAW264.7 macrophages

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Abstract

Propose: The inflammatory response of macrophages is regulated by vitamin E isomers and metabolites. The long-chain metabolite α-13′-carboxychromanol (α-13'-COOH) is formed by hepatic α-TOH catabolism and acts as a regulatory metabolite via pathways that are different from its metabolic precursor.

Methods: Using semi-synthetically-derived α-13'-COOH we profiled expression of pro- and anti-inflammatory genes by RT-qPCR and of key proteins by Western blotting. Effects of α-13'-COOH on inflammatory burst were assessed by measuring production of nitric oxide and prostaglandin (PG) E_2 via enzymatically assays and ELISA.

Results: α-13'-COOH inhibits pro-inflammatory pathways and the inflammatory burst in lipopolysaccharide (LPS)-stimulated murine RAW264.7 macrophages more efficiently than its metabolic precursor α-TOH. Profiling inflammation-related genes showed significant blocking of LPS induced interleukin (Il) 1β by the metabolite and its precursor as well, while upregulation of Il6 was not regulated. However, induction of Il10, cyclooxygenase 2 (Cox2) and inducible nitric oxide synthase (iNos) by LPS was significantly and more efficiently reduced by α-13'-COOH. Likely due to diminished upregulation of Cox2 and iNos mRNA and protein by more than 70% the LPS-stimulated formation of nitric oxide and PGE_2, was significantly reduced by α-13'-COOH. Interestingly, α-13'-COOH acts independently from translocation of the NFκB subunit p65.

Conclusions: Our study sheds new light on the mode of action of α-TOH on the inflammatory burst in macrophages, which may be mediated *in vivo* at least in part by its metabolite α-13'-COOH. Our data show that α-13'-COOH is a potent anti-inflammatory metabolite.

Keywords

α-Tocopherol, α-13'-COOH, inflammation, inflammatory burst, macrophages, macrophage activation

Non-standard Abbreviations and Acronyms:

α-CEHC	-	α-(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H)-1-benzopyran-2-propanoic acid
α-13'-COOH	-	α-13'-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid
α-LCM	-	α-tocopherol long-chain metabolite
α-TOH	-	α-tocopherol

Introduction

The inflammatory response of macrophages is a key initiator and driving force for many age-related diseases such as atherosclerosis [1]. Activated macrophages excessively accumulate lipids which leads to foam cell formation and the release of signaling molecules, such as chemokines, pro-inflammatory cytokines (tumor necrosis factor (TNF) α, interleukin (IL) 1β and IL6), nitric oxide and prostaglandins (PG) [2]. The inflammatory response is mainly triggered by the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Activation of NFκB and the translocation of its p65 subunit to the nucleus is mediated by extracellular stimuli, such as lipopolysaccharide (LPS), resulting in ubiquitination and phosphorylation of the regulatory protein IκB subunit by IκB kinase (IKK) [3]. Subsequent inflammatory enzymes, such as cyclooxygenases (COX) and inducible nitric oxide synthases (iNOS), are activated. Whereas COX1 is constitutively expressed COX2 is induced under acute and chronic inflammatory conditions and generates pro-inflammatory eicosanoids, including PGE_2. Nitric oxide synthases comprise a family of three isoforms of which the inducible NOS (iNOS) catalysis the formation of large amounts of nitric oxide from L-arginine.

Vitamin E isomers differ by methylation patterns of the hydroxychromanol ring and saturation of the side-chain (α-, β-, γ-, δ-tocopherol and -tocotrienol) [4]. Within the group of vitamin E, α-tocopherol (α-TOH) is assigned as the most active vitamer known for its anti-oxidative and anti-oxidative independent properties. α-Tocopherol regulates expression of genes, e. g. of proteins involved in uptake, transport, degradation and excretion of tocopherols, such as α-tocopherol transfer protein (α-TTP) and cytochrome P450 (CYP) 3A4 [5] and/or CYP4F2 [6], multidrug resistance (MDR) 1 [7], lipoprotein uptake, such as cluster of differentiation (CD) 36 [8], inflammation and regulation of signal transduction, e.g. via peroxisome proliferator-activated receptor (PPAR)γ [9].

In the liver, α-TOH is metabolized by side-chain truncation initiated by CYP4F2/CYP3A4-dependent ω-hydroxylation [10]. Subsequent α-oxidation in peroxisomes forms the

carboxychromanol α-13'-COOH which occurs in human serum as recently shown by our group [8]. Further β-oxidation cycles in peroxisomes and mitochondria result in the formation of α-carboxyethyl-hydroxychroman (α-CEHC[1]), the main catabolic end-product of α-TOH metabolism [11,12].

The inhibition of the inflammatory response by tocopherol and tocotrienol isomers has been investigated intensively, but the effects of α-LCM remain poorly understood. Jiang *et al.* reported that 9'-COOH and 13'-COOH inhibit COX activity in epithelial A549 cells [13]. We therefore investigated the modulation of the inflammatory response of murine RAW264.7 macrophages by the physiological α-LCM α-13'-COOH. Further we analyzed the underlying signal transduction processes. Our results reported here are in good agreement with recently published data by our group that characterize α-13'-COOH as a member of a new class of signaling molecule that shows higher bioactivity than α-TOH [8]. We therefore assume a new molecular mode of action of α-TOH via its LCM α-13'-COOH in regulating the inflammatory response of macrophages.

[1] α-CEHC, α-(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H)-1-benzopyran-2-propanoic acid

Materials and Methods

Chemicals

If not indicated otherwise chemicals were obtained from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), or Merck Millipore (Darmstadt, Germany).

RAW264.7 macrophage culture

Murine RAW264.7 macrophages (ATCC, Manassas, VA) were cultivated in 150 cm^2 cell culture flasks in high glucose (4.5 g/l) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 0.1 mg/ml penicillin/streptomycin/L-glutamine mixture. For further culturing, cells were scraped from the cell culture flask and split in a mixture of 70% fresh high glucose DMEM and 30% used culture DMEM obtained from previous periods of RAW264.7 macrophage culture. Cells were split trice a week at a confluence of about 80% and cultured at 37°C in humidified 5% CO_2/95% air atmosphere. For experiments cells were seeded, cultured for 24 h and then incubated with compounds as indicated in the figures. Cells were harvested for further processing as described below.

RNA isolation and cDNA synthesis

Total RNA was isolated from cell lysates using Qiagen RNeasy Mini kit (Hilden, Germany) including on-column DNase I digestion (Qiagen) as described earlier [14]. cDNA synthesis was performed using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), 5 µg of total RNA and 500 ng/µl oligo-dT primers as outlined previously [15].

Quantitative real-time RT-PCR (RT-qPCR)

Quantitative real-time RT-PCR was performed on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using QuantiTect SYBR® Green PCR kit (Qiagen) as reported earlier [15]. Primers (Il6, Il1β, Il10, Tnfα, iNos, Cox2 and Ppib; see Table 1) were purchased from Invitrogen (Karlsruhe, Germany). The PCR runs included a 15 min pre-incubation at 95°C, followed by a 40 cycle two-step PCR consisting of a denaturing phase at

94°C for 15 s and a combined annealing and extension phase at 60°C for 30 s. Results were analyzed using LightCycler software 1.5.0.39.

Immunoblotting

iNos and Cox2

Cells were harvested using a non-denaturing buffer (50 mM Tris-HCl, 0.5% Nonidet P40, 250 mM NaCl, 15 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4) containing 1% protease inhibitor (Fisher Scientific, Schwerte) and mixed 3:1 with loading buffer 3:1 (6.26% 1 M Tris-HCl, 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue). Cellular proteins were separated by SDS-PAGE and transferred to PVDF membranes (VWR, Darmstadt, Germany) using a transfer buffer containing 0.25 M Tris, 1.92 M glycine, 0.1% SDS and 20% methanol (pH 8.3). For iNos and Cox2 antibodies were incubated in SignalBoost™ Immunreaction Enhancer kit (Calbiochem, Darmstadt, Germany), and for α-tubulin a hybridization puffer containing 0.5% milk powder and PBS (0.137 M NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4 x 2 H_2O, 1.5 mM KH_2PO_4, pH 7.4) were used, respectively. Primary antibodies mouse anti-iNOS (clone 6), rabbit anti-COX-2 (clone EP1978Y) and mouse anti-α-tubulin (clone B-5-1-2) were from BD Biosciences (Heidelberg, Germany), Epitomics (Burlingame, CA), and Sigma-Aldrich, respectively. Secondary antibodies (rabbit anti-mouse and swine anti-rabbit both labeled with horseradish peroxidase) were from DAKO (Hamburg, Germany).

NFκB

Cells were scraped in warm PBS, sedimented and resuspended in non-denaturating buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.025% NP-40) containing 2% protease inhibitor. For sub-cellular fractionation cells were vortexed and incubated on ice. Aliquots of the total fraction were taken and samples were then centrifuged. The supernatant containing the cytosolic fraction and the pellet with the nuclear fraction were separated. Both fractions were resuspended in non-denaturating buffer (50 mM Tris-HCl, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 1 mM EDTA). All fractions were sonicated and mixed with loading buffer. Proteins were separated by SDS-PAGE and transferred to PVDF membranes

as outlined above. Primary antibodies against NFκB subunit p65 (rabbit anti-p65, clone E379) from Epitomics, mouse anti-α-tubulin (clone B-5-1-2) and rabbit anti-PARP (clone 46D11) from Cell Signaling Technology (Danvers, MA) were used. Secondary antibodies (rabbit anti-mouse or swine anti-rabbit labeled with horseradish peroxidase) were from DAKO. For p65 and PARP the SignalBoost™ Immunreaction Enhancer kit was used.

Detection

For detection Pierce ECL Western Blotting Substrate and CL-XPosure™ Films (Thermo Scientific, Rockford, IL) were applied. Blots were analyzed densitometrically using ImageJ software version 1.4.3.67.

Quantification of nitric oxide formation using Griess assay

RAW264.7 macrophages were seeded in a mixture of 70% fresh supplemented high glucose DMEM and 30% used culture medium for 24 h. For experiments, adherent cells were washed twice and incubated with either 20 µM α-TOH or 5 µM α-13'-COOH in fresh serum free high glucose DMEM for further 24 h followed by another 24 h incubation of both 100 ng/ml LPS and either solvent, 20 µM α-TOH or 5 µM α-13'-COOH. For Griess assays the cell culture supernatants were removed and centrifuged to remove cells. The supernatants were diluted with water (1:0.87), mixed with Griess reagent (15 %) and incubated in the dark. The concentration of nitrite in the supernatants was measured by 544 nm using a BMG Labtech FLUOstar omega and MARS data analysis software version 2.41.

Prostaglandin E_2 quantification via ELISA

RAW264.7 macrophages were seeded in supplemented high glucose DMEM as described above for 24 h and incubated with either solvent, 5 µM α-13'-COOH or 100 µM α-TOH for 24 h followed by another 24 h of co-incubation with 100 ng/ml LPS and either 5 µM α-13'-COOH or 100 µM α-TOH. Next, cell culture supernatants were collected and immediately stored on ice until centrifugation for removing cells and debris. Release of PGE_2 by macrophages was quantified using the Prostaglandin E_2 Enzyme Immunoassay Kit (Biotrend, Cologne) as

indicated in the manufacturer's protocol. In brief, 100 µl of each sample or standard were mixed with 100 µl assay buffer. The samples were then pipetted in microtiter plate wells coated with goat anti-mouse IgG. 25 µl PGE_2 peroxidase conjugates and 25 µl monoclonal mouse PGE_2 antibody solution were added. If necessary, supernatants were diluted to comply with the linear measurement range of the assay. The sealed plate was shaken for 2 h at room temperature. After incubation the plate was washed four times with washing buffer, dried by tapping out the liquid and incubated with 100 µl tetramethylbenzidine substrate per well for 30 min. The reaction was stopped by adding 50 µl Stop Solution (Biotrend) per well. The concentration of PGE_2 in the supernatants was measured by 450 nm in a Multiscan^{TM} GO Microplate Spectrophotometer (Thermo Scientific). To exclude unspecific binding signals absorption of blanks was subtracted from that of samples. The amount of PGE_2 in the supernatants was calculated using a standard curve.

Semi-synthesis of α-LCMs

All chemicals were used as received from the supplier. Isolation of the educt garcinoic acid from the African bitter nut *Garcinia kola* and synthesis of the α-LCM were performed as previously described [16,17].

Statistics

Data are presented either as means ± standard deviation or as means ± standard error of the mean (SEM) of independent experiments as indicated. In order to test for statistical significance paired Student's t-tests were performed using Microsoft Excel 2007/2010.

Results

It has been quite well studied that the inhibitory capacity of tocopherols and tocotrienols as well as their modes of action on pro-inflammatory pathways depends on the isomer [13]. For example, cyclooxygenase activity is inhibited *in vitro* by α- and β-TOH, γ-TOH, γ-tocotrienol and δ-TOH with decreasing effectiveness. Beside the isomers, the carboxychromanol metabolites δ- and γ-9'-COOH as well as δ- and γ-13'-COOH are more potent anti-inflammatory agents compared to their metabolic tocopherol precursors δ-TOH and γ-TOH in inhibiting COX1 and 2 activity [13]. We therefore analyzed the anti-inflammatory action of α-13'-COOH, a physiological α-LCM recently identified by our group in human serum [8]. Studies in THP-1 macrophages defined an EC_{50} value of 7.4 µM ± 1.5 µM for α-13'-COOH but no cytotoxicity was observed for α-TOH up to 100 µM using sulforhodamine B assays [8]. Therefore we used 5 µM of α-13'-COOH that was obtained semi-synthetically [17] and a maximum of 100 µM of α-TOH for the functional studies on RAW264.7 mouse macrophages outlined here. If not indicated otherwise cells were pre-incubated with α-13'-COOH or α-TOH for 24 h prior to co-incubation of the compounds with 100 ng/ml LPS for another 24 h. We investigated α-13'-COOH in comparison to α-TOH in all experiments in order to get first insights in the importance of the side-chain oxidation for the biological function and effectiveness of the tocopherol metabolite.

Lipopolysaccharide-induced upregulation of Il1β, Il10 and Tnfα mRNAs but not of Il6 is blocked by α-13'-COOH

Previous studies have shown that α-TOH inhibits the inflammatory response of macrophages by regulating the inflammatory response. We therefore investigated the effect of α-13'-COOH on the LPS-induced inflammatory response of mouse RAW264.7 macrophages. To analyze the effect of α-13'-COOH on the expression of typical LPS response genes, we first focused on Il1β and Tnfα mRNA as pro-inflammatory mediators, on Il6 as an ambivalent inflammatory mediator and Il10 as an anti-inflammatory one. As shown in Fig. 1, neither 5 µM α-13'-COOH nor 100 µM α-TOH had any effects on the expression of the four selected marker genes. As

expected, LPS induced significantly the expression of Il1β (2,000-fold, $p < 0.001$), Il6 (12,000 to 16,500-fold, $p < 0.001$), and Tnfα (15 to 34-fold, $p < 0.001$) as well as Il10 (23 to 15-fold, $p < 0.05$). Treatment with neither 5 µM α-13'-COOH nor with 100 µM α-TOH affected the LPS-induced expression of Il6 (Fig. 1A). α-13'-COOH but not α-TOH tended to decrease LPS-induced expression of Tnfα (Fig. 1B). In contrast, LPS-induced upregulation of Il1β was significantly reduced by both, α-13'-COOH and α-TOH by 94% and 61%, respectively ($p < 0.05$, $p < 0.01$; Fig. 1C). Further, α-13'-COOH blocked LPS-induced upregulation of Il10 mRNA by 89% ($p < 0.05$), whereas α-TOH had no effect (Fig. 1D).

Lipopolysaccharide-induced upregulation of Cox2 and iNos is blocked by α-13'-COOH but not by α-TOH

Expression of Cox2 and iNos is also induced by LPS and α-TOH has been considered to modulate this upregulation. Studies analyzing the upregulation of LPS-induced Cox2 expression by α-TOH differ in their results; while Yam *et al.* found that all vitamin E isomers except α-TOH downregulated LPS-induced Cox2 gene expression in murine RAW264.7 macrophages, Ng and coworkers showed that LPS-induced Cox2 gene expression was inhibited by α-TOH in peritoneal macrophages [3,18]. On the other hand, upregulation of iNos by LPS is consistently decreased by α-TOH [3]. We therefore wanted to know whether α-13'-COOH modulates the response of Cox2 and iNos to LPS in RAW264.7 macrophages. Neither α-13'-COOH nor α-TOH influenced the expression of Cox2 and iNos at the mRNA and protein level in mouse RAW264.7 macrophages in our model (Fig. 2). LPS significantly increased the expression of both Cox2 mRNA (3,200-fold, $p < 0.001$; Fig. 2A) and iNos mRNA (250-fold, $p < 0.001$; Fig. 2B). α-13'-COOH decreased the LPS-induced upregulation of Cox2 mRNA by 84% ($p < 0.01$) and iNos mRNA by 92% ($p < 0.001$); thus, α-13'-COOH was even more effective at lower concentrations than α-TOH which tended to block the LPS-induced upregulation of Cox2 mRNA by 59% and iNos mRNA by 37%, respectively (Fig. 2A, 2B). Pre-incubation of α-13'-COOH for 24 h followed by another 24 h of co-incubation of α-13'-COOH and 100 ng/ml LPS did not reveal any regulatory effects of α-13'-COOH on LPS-induced Cox2 protein levels (data not shown). However, LPS-upregulated Cox2 protein

levels were blocked significantly after 14 h of co-incubation of LPS and α-13'-COOH by 75% ± 24% ($p < 0.001$; Fig. 2D), while upregulation of iNos protein by LPS was significantly inhibited by α-13'-COOH after 24 h by 74% ± 2% ($p < 0.001$; Fig. 2E). Neither upregulation of Cox2 protein nor iNos protein by LPS was affected by α-TOH.

Formation of PGE_2 and nitric oxide in RAW264.7 macrophages is blocked by α-13'-COOH

Cyclooxygenases 2 and iNos synthesize the signaling molecules PGE_2 and nitric oxide, which play a key role in the LPS-induced immune response of macrophages. As shown in Fig. 2, expression of Cox2 and iNos is down-regulated at the mRNA and protein level by the α-LCM α-13'-COOH. Hence, we investigated the effect of α-13'-COOH on the LPS-stimulated production of PGE_2 and nitric oxide by Cox2 and iNos, respectively, in RAW264.7 macrophages. Treatment with 100 ng/ml LPS significantly induced the release of PGE_2 by RAW264.7 up to 18.3 ng/ml ± 4.1 ng/ml ($p < 0.05$). α-Tocopherol decreased the LPS-induced release of PGE_2 to 7.9 ng/ml ± 2.4 ng/ml ($p < 0.05$), and the α-LCM blocked the LPS-mediated production of PGE_2 almost to baseline (1.5 ng/ml ± 1.2 ng/ml; $p < 0.05$) as shown in Fig. 3A. We also measured the effects of α-13'-COOH and α-TOH on the formation of nitric oxide in RAW264.7 macrophages activated by LPS. Treatment with LPS increased the concentration of nitric oxide in the culture supernatant to 34.3 µM ± 4.5 µM ($p < 0.01$). In our experimental setup, we observed no significant effect of α-TOH on the LPS-triggered induction of nitric oxide production. In contrast to α-TOH, α-13'-COOH significantly decreased the LPS-induced formation of nitric oxide down to 2.7 µM ± 0.9 µM ($p < 0.001$; Fig. 3B). Neither α-TOH nor the α-13'-COOH had an effect on the basal release of nitric oxide in non-stimulated RAW264.7 macrophages.

Lipopolysaccharide-induced translocation of NFκB is not affected by α-13'-COOH

We were next interested whether the immunomodulatory properties of α-13'-COOH are mediated via the key regulator of the LPS response in macrophages, namely NFκB. Stimulation of macrophages with LPS results in the phosphorylation of the IκB subunit and

the translocation of the p50/p65 heterodimer from the cytosol into the nucleus where it activates the expression of respective target genes. As shown in Fig. 4 treatment of 1 µg/ml LPS for one hour resulted in a significant translocation of the NFκB subunit p65 from the cytosol to the nucleus while the amount of total and cytosolic p65 remained unaffected. Neither α-13'-COOH nor α-TOH caused significant changes in the total, cytosolic or nuclear amount of p65 in the absence of LPS. However, α-13'-COOH failed to block the translocation of the p65 subunit into the nucleus (Fig. 4A, left), whereas α-TOH significantly enhanced translocation of p65 by 54% ± 20% ($p < 0.05$; Fig. 4A, right) in our cell model. The total cell and cytosolic fractions did not show any changes in the amount of the p65 subunit during co-incubation with LPS and either α-13'-COOH or α-TOH (Figs. 4B and 4C). The reference proteins α-tubulin (for the cytosolic fraction and the total cell lysate) and PARP (for the nuclear fraction) remained unchanged under all conditions. Thus, α-13'-COOH exhibits its effects on the LPS response of macrophages independently from the translocation of the NFκB subunit p65.

Discussion and Conclusions

α-Tocopherol is widely used to prevent oxidation and inflammation-related diseases. Next to its antioxidant properties, vitamin E directly regulates signal transduction and expression of genes involved in inflammatory processes to different extents depending on the isomer [19].

The overwhelming release of chemokines, cytokines and mediators such as nitric oxide and PGE_2 by macrophages characterizes the inflammatory burst. Vitamin E supplementation is known to decreases cytokine production *in vivo* [20], but the modes of action by which vitamin E affects the inflammatory burst *in vivo* and *in vitro* remain poorly understood. Next to TOHs, metabolites formed during hepatic catabolism of vitamin E, such as α- and γ-CEHC, are potent inhibitors of the inflammatory burst that act by blocking the expression of iNos and Cox2 in TNFα-stimulated EOC-20 microglial cells and LPS-stimulated RAW264.7 murine macrophages [18,21]. We recently identified α-13'-COOH as a hepatic metabolite of α-TOH that occurs also in human serum of healthy non-supplemented volunteers; we recently provided first evidence that the α-LCM may represent a new class of regulatory signaling molecules [8]. We therefore wondered whether the α-LCM may affect the LPS-triggered inflammatory burst of macrophages. Measurement of selected pro- and anti-inflammatory mediators showed that α-13'-COOH affects gene expression more effectively and differently than α-TOH (Fig. 1A to 1D, 2A and 2B). Whereas α-13'-COOH inhibited the LPS-induced expression of Il1β the up-regulated expression of Il6 and Tnfα is not affected by α-13'-COOH. Since the expression of the pro-inflammatory gene Il1β is blocked by α-13'-COOH also the late response to inflammatory stimuli via the increased expression of Il10 does also not occur in the presence of α-13'-COOH.

Cyclooxygenases and iNOS catalyze signaling molecule production and play therefore an important role in regulating inflammatory processes. Down-regulation of iNos expression after α-TOH treatment has been considered by Ng *et al.* and Jiang *et al.* in LPS-stimulated murine macrophages [3,22]. However, we could not find a significant effect of α-TOH on iNos

expression (Fig. 2), possibly due to different incubation conditions used in our study. In accordance to others we confirmed that Cox2 expression is not affected by α-TOH [18,22,23]. In contrast to α-TOH, α-13'-COOH exhibits significant effects in LPS-stimulated macrophages on the expression of both iNos and Cox2 at the mRNA and protein levels (Fig. 2), which implicates a different mode of action of the α-LCM compared to α-TOH.

In good agreement with the inhibition of iNos and Cox2 expression the formation of PGE_2 and nitric oxide is also efficiently blocked by α-13'-COOH; interestingly this takes place at lower concentrations than with α-TOH. However, α-TOH significantly blocks PGE_2 but not nitric oxide formation. For PGE_2 and nitric oxide different α-TOH concentrations (100 μM vs. 20 μM) were used since higher amounts of α-TOH interfered with the Griess assay. Since α-TOH is less efficient in blocking nitric oxide formation compared to other isomers of vitamin E, the structure of the chromanol ring system seems to be important for this [3,18,20]. In addition, the short-chain metabolites α-CEHC and γ-CEHC are more potent in inhibiting nitric oxide production compared to the metabolic precursor itself [21]. The α-TOH analogue trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which differs from α-CEHC in length and oxidation of the carboxylate tail, is ineffective in inhibiting nitric oxide formation in EOC-20 cells [21]. Since α-13'-COOH significantly blocks the LPS-induced release of PGE_2 nearly to baseline levels (Fig.3), we conclude that the oxidative modification of the side-chain is as important for the inhibition as the chromanol ring.

Although the expression of Cox2 was not regulated by α-TOH in our study we showed a significant blocking of the release of PGE_2 in murine RAW264.7 macrophages in response to LPS (Fig. 3A). A closer look to the literature reveals contrary results on inhibitory effects of α-TOH on the release of PGE_2 release which may depend on the cell type [18,20,23]. Studies of non-α-TOH isomers and metabolites are more consistent with respect to the inhibition of PGE_2 production [13,18,20,24]. Traber *et al.* already postulated that the ability of α-TOH metabolites to block the synthesis of PG may depend on the length of the phytyl side chain. Hence middle and short-chain metabolites of α-TOH were highly effective in blocking the

synthesis of PGE_2 in response to LPS due to their higher cell membranes penetration mobility [25]. Further, Jiang *et al.* showed that δ-13'-COOH and γ-13'-COOH inhibit the COX2-catalyzed production of PGE_2 in IL1β-stimulated human lung adenocarcinoma A549 cells [13]. However, we found significant inhibitory effects of α-LCM α-13'-COOH on the release of PGE_2 in LPS-stimulated murine RAW264.7 macrophages. With these results we showed for the first time the inhibition of the LPS-stimulated release of nitric oxide and PGE_2 by α-13'-COOH. At present, we do not know whether the oxidation of the side-chain of α-13'-COOH affects its membrane mobility; but our study provides clear evidence for the regulatory features of this metabolite.

Inflammatory pathways are strongly regulated by activation of NFκB which mediates its effects by DNA binding following translocation of the p50/p65 heterodimer from the cytosol into the nucleus [26]. Treatment with α-TOH has been shown to inhibit the activity of NFκB depending on the cell type [20,27–30]. Using murine RAW264.7 macrophages we found enhanced LPS-induced translocation of the NFκB subunit p65 after treatment with α-TOH (Fig. 4A). Protein kinase C (PKC) phosphorylates IκB which results in detachment of IκB from the p50/p65 heterodimer and finally in the translocation of the p50/p65 heterodimer into the nucleus. It has been shown that NFκB is inactivated via PKC inhibition by α-TOH in vascular smooth muscle cells [31]. Since our results do not confirm this, other regulatory pathways may mediate the effects of α-TOH on NFκB in RAW264.7 macrophages. Regulation of NFκB activity by metabolites and derivatives of α-TOH, namely 2,2,5,7,8-pentamethyl-6-hydroxychromane and α-tocopheryl succinate, has been described [29]. Our results reported here clearly show that the potent anti-inflammatory activity of α-13'-COOH on the expression and activity of Cox2 and iNos is independent from the translocation of the NFκB subunit p65 (Fig. 4A). This again provides evidence that the regulatory pathways affected by α-13'-COOH are different form that of α-TOH.

In summary, our findings clearly demonstrate that α-13'-COOH modulates the inflammatory response of macrophages more potently and via signaling pathways different from that of α-

TOH. Thus we present here further hints for a physiological role of the metabolite α-13'-COOH. Finally, our data show that the mode of action of α-TOH may be much more complex due to the regulatory functions of the metabolite α-13'-COOH. Further investigations are therefore required to elucidate the *in vivo* relevance of our findings.

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Disclosures

None.

References

[1] S. Pant, A. Deshmukh, G.S. GuruMurthy, N.V. Pothineni, T.E. Watts, F. Romeo, J.L. Mehta, Inflammation and Atherosclerosis--Revisited, J Cardiovasc Pharmacol Ther (2013).

[2] A. Nguyen, H. Tao, M. Metrione, T. Hajri, Very low density lipoprotein receptor expression is a determinant factor in adipose tissue inflammation and adipocyte-macrophage interaction, J Biol Chem (2013).

[3] L.-T. Ng, H.-J. Ko, Comparative effects of tocotrienol-rich fraction, α-tocopherol and α-tocopheryl acetate on inflammatory mediators and nuclear factor kappa B expression in mouse peritoneal macrophages, Food Chem 134 (2012) 920-925.

[4] D.J. Mustacich, S.W. Leonard, N.K. Patel, M.G. Traber, Alpha-tocopherol beta-oxidation localized to rat liver mitochondria, Free Radic Biol Med 48 (2010) 73-81.

[5] N. Landes, P. Pfluger, D. Kluth, M. Birringer, R. Rühl, G.-F. Böl, H. Glatt, R. Brigelius-Flohé, Vitamin E activates gene expression via the pregnane X receptor, Biochem Pharmacol 65 (2003) 269-273.

[6] S.M. Farley, S.W. Leonard, A.W. Taylor, M. Birringer, K.Z. Edson, A.E. Rettie, M.G. Traber, ω-Hydroxylation of phylloquinone by CYP4F2 is not increased by α-tocopherol, Mol Nutr Food Res (2013).

[7] D.J. Mustacich, K. Gohil, R.S. Bruno, M. Yan, S.W. Leonard, E. Ho, C.E. Cross, M.G. Traber, Alpha-tocopherol modulates genes involved in hepatic xenobiotic pathways in mice, J Nutr Biochem 20 (2009) 469-476.

[8] M. Wallert, S. Mosig, K. Rennert, H. Funke, M. Ristow, R.M. Pellegrino, G. Cruciani, F. Galli, S. Lorkowski, M. Birringer, Long-chain metabolites of α-tocopherol occur in human serum and inhibit macrophage foam cell formation in vitro, Free Radic Biol Med 68 (2013 // 2014) 43-51.

[9] R. Ricciarelli, J.M. Zingg, A. Azzi, Vitamin E reduces the uptake of oxidized LDL by inhibiting CD36 scavenger receptor expression in cultured aortic smooth muscle cells, Circulation 102 (2000) 82-87.

[10] S.E. Campbell, W.L. Stone, S.G. Whaley, M. Qui, K. Krishnan, Gamma (gamma) tocopherol upregulates peroxisome proliferator activated receptor (PPAR) gamma (gamma) expression in SW 480 human colon cancer cell lines, BMC Cancer 3 (2003) 25.

[11] A. Azzi, R. Gysin, P. Kempná, A. Munteanu, Y. Negis, L. Villacorta, T. Visarius, J.-M. Zingg, Vitamin E mediates cell signaling and regulation of gene expression, Ann N Y Acad Sci 1031 (2004) 86-95.

[12] M. Birringer, D. Drogan, R. Brigelius-Flohé, Tocopherols are metabolized in HepG2 cells by side chain omega-oxidation and consecutive beta-oxidation, Free Radic Biol Med 31 (2001) 226-232.

[13] Q. Jiang, X. Yin, M.A. Lill, M.L. Danielson, H. Freiser, J. Huang, Long-chain carboxychromanols, metabolites of vitamin E, are potent inhibitors of cyclooxygenases, Proc Natl Acad Sci U.S.A. 105 (2008) 20464-20469.

[14] K. Stolle, M. Schnoor, G. Fuellen, M. Spitzer, P. Cullen, S. Lorkowski, Cloning, genomic organization, and tissue-specific expression of the RASL11B gene, Biochim Biophys Acta 1769 (2007) 514-524.

[15] K. Stolle, M. Schnoor, G. Fuellen, M. Spitzer, T. Engel, F. Spener, P. Cullen, S. Lorkowski, Cloning, cellular localization, genomic organization, and tissue-specific expression of the TGFbeta1-inducible SMAP-5 gene, Gene 351 (2005) 119-130.

[16] K. Terashima, T. Shimamura, M. Tanabayashi, M. Aqil, J.A. Akinniyi, M. Niwa, Constituents of the Seeds of Garcinia Kola: Two New Antioxidants, Garcinoic Acid and Garcinal, Heterocycles 45 (1997) 1559-1566.

[17] M. Birringer, D. Lington, S. Vertuani, S. Manfredini, D. Scharlau, M. Glei, M. Ristow, Proapoptotic effects of long-chain vitamin E metabolites in HepG2 cells are mediated by oxidative stress, Free Radic Biol Med 49 (2010) 1315-1322.

[18] M.-L. Yam, Abdul Hafid, Sitti Rahma, H.-M. Cheng, K. Nesaretnam, Tocotrienols Suppress Proinflammatory Markers and Cyclooxygenase-2 Expression in RAW264.7 Macrophages, Lipids 44 (2009) 787-797.

[19] J.-M. Zingg, Modulation of signal transduction by vitamin E, Mol Aspects Med 28 (2007) 481-506.

[20] J.M. Cook-Mills, C.A. McCary, Isoforms of vitamin E differentially regulate inflammation, Endocr Metab Immune Disord Drug Targets 10 (2010) 348-366.

[21] P. Grammas, L. Hamdheydari, E.J. Benaksas, S. Mou, Q.N. Pye, W.J. Wechter, R.A. Floyd, C. Stewart, K. Hensley, Anti-inflammatory effects of tocopherol metabolites, Biochem Bioph Res Co 319 (2004) 1047-1052.

[22] Q. Jiang, I. Elson-Schwab, C. Courtemanche, B.N. Ames, gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells, Proc Natl Acad Sci U.S.A. 97 (2000) 11494-11499.

[23] S. Kim, E.-H. Lee, S.H. Kim, S. Lee, S.-J. Lim, Comparison of three tocopherol analogs as an inhibitor of production of proinflammatory mediators in macrophages, J Pharmacol Sci 118 (2012) 237-244.

[24] Q. Jiang, B.N. Ames, Gamma-tocopherol, but not alpha-tocopherol, decreases proinflammatory eicosanoids and inflammation damage in rats, FASEB J. 17 (2003) 816-822.

[25] M.G. Traber, L. Packer, Vitamin E: beyond antioxidant function, Am J Clin Nutr 62 (1995) 1501S-1509S.

[26] H. Buss, A. Dörrie, M.L. Schmitz, R. Frank, M. Livingstone, K. Resch, M. Kracht, Phosphorylation of serine 468 by GSK-3beta negatively regulates basal p65 NF-kappaB activity, J Biol Chem 279 (2004) 49571-49574.

[27] B. Ekstrand-Hammarström, C. Osterlund, B. Lilliehöök, A. Bucht, Vitamin E down-modulates mitogen-activated protein kinases, nuclear factor-kappaB and inflammatory responses in lung epithelial cells, Clin Exp Immunol 147 (2007) 359-369.

[28] K.N. Islam, S. Devaraj, I. Jialal, alpha-Tocopherol enrichment of monocytes decreases agonist-induced adhesion to human endothelial cells, Circulation 98 (1998) 2255-2261.

[29] Y.J. Suzuki, L. Packer, Inhibition of NF-kappa B activation by vitamin E derivatives, Biochem Biophys Res Commun 193 (1993) 277-283.

[30] R. Faruqi, de la Motte, C, P.E. DiCorleto, Alpha-tocopherol inhibits agonist-induced monocytic cell adhesion to cultured human endothelial cells, J Clin Invest 94 (1994) 592-600.

[31] A. Azzi, The role of alpha-tocopherol in preventing disease, Eur J Nutr 43 Suppl 1 (2004) I/18-25.

Figures and Figure legends

Figure 1

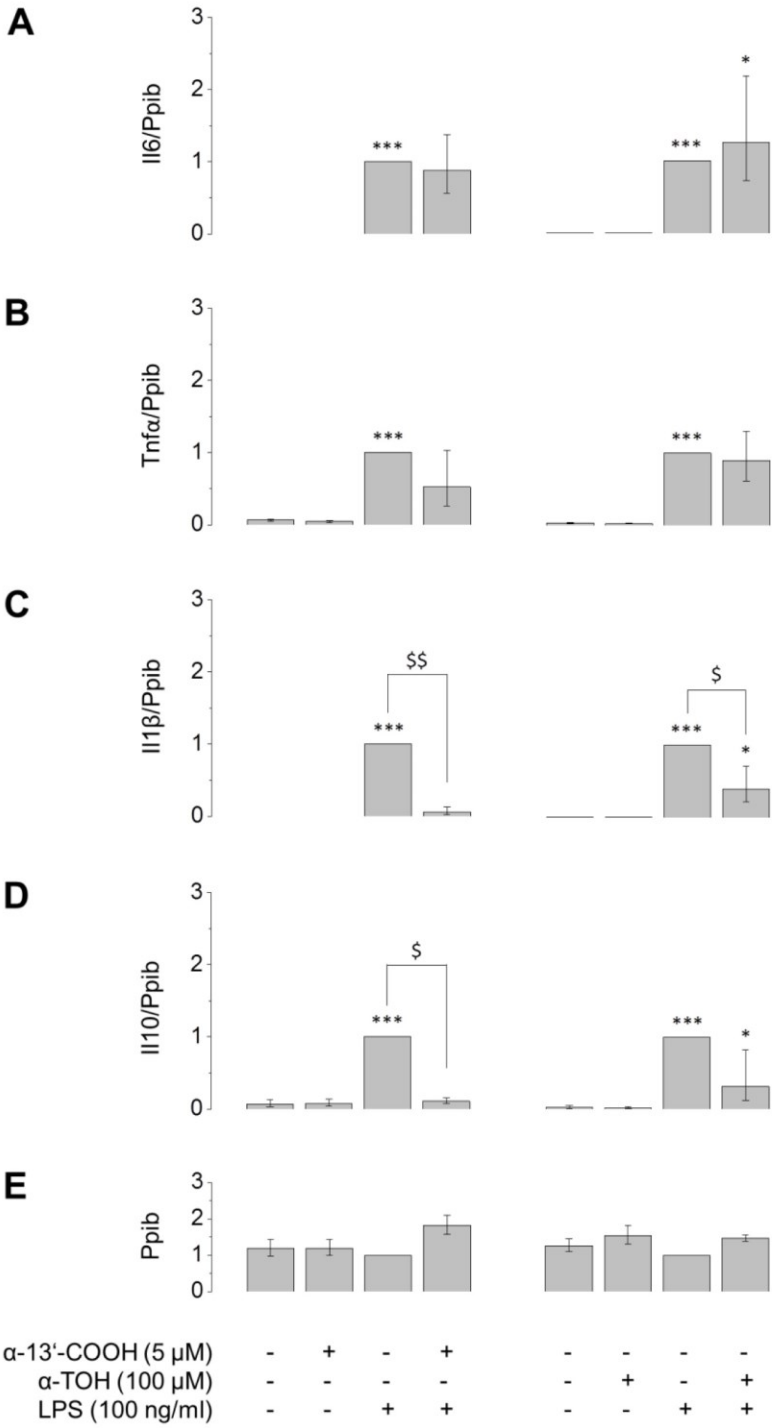

Figure 1: α-13'-COOH differently affects the expression of LPS response genes in mouse RAW264.7 macrophages. Murine RAW264.7 macrophages were pre-incubated with either solvent, 5 µM α-13'-COOH (left) or 100 µM α-TOH (right) for 24 h followed by co-incubation of 100 ng/ml LPS and either solvent, 5 µM α-13'-COOH or 100 µM α-TOH for another 24 h. Appropriate control macrophages were cultured in the presence of either solvent or compound only for the same time periods; samples cultured for 24 h in the presence of solvent followed by another 24 h with LPS served as reference and were defined as 1. Expression levels of the inflammatory response genes Il6, Tnfα, Il1β and Il10 were measured using RT-qPCR and normalized to the expression of the reference gene Ppib. **(A)** The α-LCM α-13'-COOH and its precursor α-TOH did not block LPS-induced upregulation of Il6 mRNA levels. **(B)** But LPS-induced expression of Tnfα mRNA tended to be decreased by α-13'-COOH by 48%, whereas α-TOH showed no effect. **(C)** LPS-induced expression of Il1β mRNA was significantly blocked by α-13'-COOH and α-TOH by 94% ($p < 0.01$) and 61% ($p < 0.05$), respectively. **(D)** The induction of Il10 expression by LPS was significantly reduced by α-13'-COOH by 89% ($p < 0.05$), and to 19% by α-TOH. Expression of the reference gene Ppib remained unchanged under all conditions. Error bars display calculated maximum and minimum expression levels representing SEM expression levels of four to five independent biological experiments, each measured in one technical replicate. *, $p < 0.05$; ***, $p < 0.001$ (*vs*. solvent control); $^{\$}$, $p < 0.05$; $^{\$\$}$, $p < 0.01$ (*vs*. LPS treatment).

Figure 2

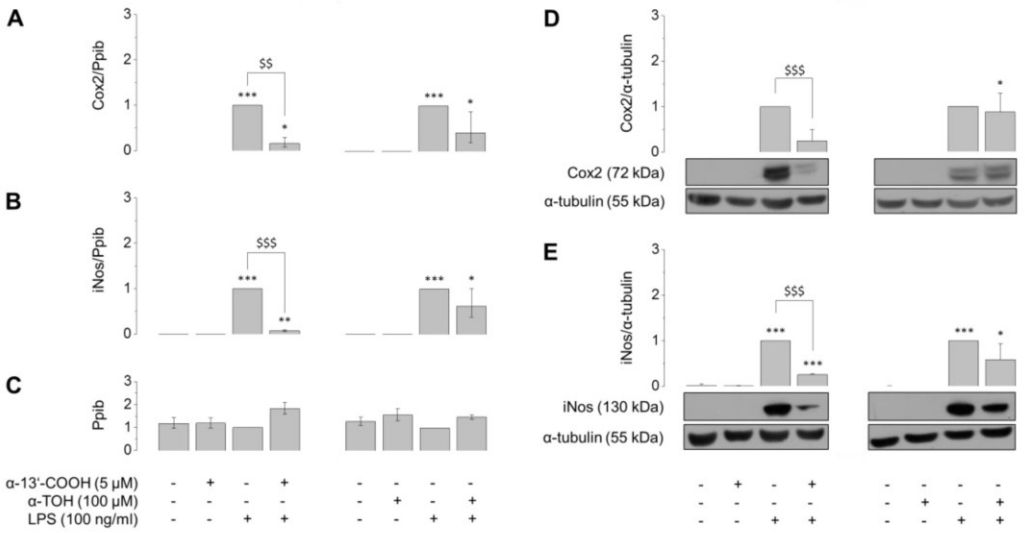

Figure 2: Lipopolysaccharide-induced expression of Cox2 and iNos is inhibited by α-13'-COOH in mouse RAW264.7 macrophages. Murine RAW264.7 macrophages were pre-incubated with solvent, 5 µM α-13'-COOH (left) or 100 µM α-TOH (right) for 24 h followed by co-incubation of 100 ng/ml LPS and either solvent, 5 µM α-13'-COOH or 100 µM α-TOH for another 24 h to perform RT-qPCR analyses **(A-C)** or for 14 h (Cox2) or 24 h (iNos) for Western blot analyses **(D,E)**. Appropriate control cells were cultured in the presence of either solvent or compound only for 24 h; samples cultured for 24 h in the presence of solvent followed by another 24 h **(A-C, E)** or for 14 h **(D)** with LPS served as reference and were defined as 1. Messenger RNA levels were normalized to Ppib mRNA and protein levels were normalized to α-tubulin for histogram quantification of Western blots. Lipopolysaccharide-induced upregulation of **(A)** Cox2 mRNA and **(B)** iNos mRNA was significantly reduced by α-13'-COOH by 84% (SEM min 7%, SEM max 14%, $p < 0.01$) and 92% (SEM min 1%, SEM max 2%, $p < 0.001$), respectively; α-TOH tended to reduce the upregulation of Cox2 and iNos by LPS by 59% (SEM min 22%, SEM max 46%) and 37% (SEM min 24%, SEM max 39%), respectively. Expression of Ppib remained unchanged under all conditions. Lipopolysaccharide-induced Cox2 protein levels **(D)** and iNos protein levels **(E)** were significantly reduced by α-13'-COOH by 75% ± 24% ($p < 0.001$) and 74% ± 2% ($p < 0.001$),

respectively, but not by α-TOH (12% ± 41% and 41% ± 35%) compared to LPS-stimulated cells. Expression of α-tubulin remained unchanged under all conditions. The Western blots are representative examples of the blots used for densitometry. **(A-C)** Error bars display calculated maximum and minimum expression levels representing SEM expression levels of four to five independent biological experiments, each measured in one technical replicate. **(D,E)** Means of three independent biological experiments measured in one (iNos) or two (Cox2) technical replicates are shown; error bars represent standard deviations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (*vs.* control); $^{\$\$}$, $p < 0.01$, $^{\$\$\$}$, $p < 0.001$ (*vs.* LPS treatment).

Figure 3

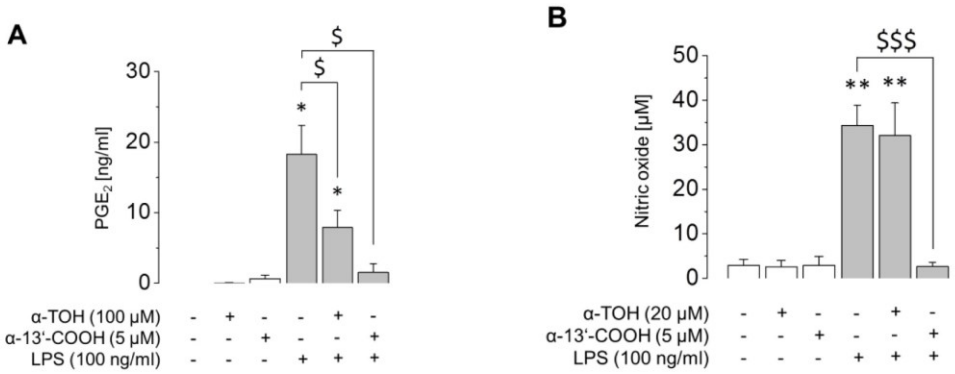

Figure 3: Release of PGE_2 and nitric oxide in LPS-stimulated mouse RAW264.7 macrophages is blocked by α-13'-COOH. RAW264.7 macrophages were pre-incubated for 24 h with solvent, 5 µM α-13'-COOH or **(A)** 100 µM or **(B)** 20 µM α-TOH cells followed by an incubation with 100 ng/ml LPS (grey bars) in combination with either solvent, 5 µM α-13'-COOH or α-TOH (20 or 100 µM) for further 24 h. Appropriate control cells were cultured in the presence of either solvent or compound only for 24 h (white bars). **(A)** Basal release of PGE_2 remained almost unaffected by both α-13'-COOH or α-TOH. Treatment with 100 ng/ml LPS significantly induced PGE_2 levels in the supernatant of RAW264.7 macrophages up to 18.3 ± 4.1 ng/ml ($p < 0.05$). Co-incubation with α-TOH decreased PGE_2 concentration to 7.9 ± 2.4 ng/ml ($p < 0.05$). α-13'-COOH reduced the levels of PGE_2 nearly to baseline levels of 1.5 ± 1.2 ng/ml ($p < 0.05$). Release of PGE_2 into the cell culture supernatants was measured in three independent experiments by ELISA. Error bars represent standard deviations. **(B)** RAW264.7 macrophages were cultured as in **(A)**. Culture supernatants were used for quantifying the release of nitric oxide using Griess assays. Basal nitric oxide levels were not affected by α-13'-COOH and α-TOH. Treatment with LPS increased the formation of nitric oxide significantly up to 34.3 µM ± 4.5 µM ($p < 0.01$). The LPS-induced increase of nitric oxide release was significantly blocked by α-13'-COOH down to almost baseline levels (2.7 µM ± 0.9 µM, $p < 0.001$), but not significantly by α-TOH (32.1 µM ± 7.4 µM). Means of **(A)** three or **(B)** four independent biological experiments performed each in **(A)** one or **(B)** four

replicates are shown; error bars display standard errors. *, $p < 0.05$, **, $p < 0.01$ (*vs*. control); $^{\$}$, $p < 0.05$, $^{\$\$\$}$, $p < 0.001$ (*vs*. LPS treatment).

Figure 4

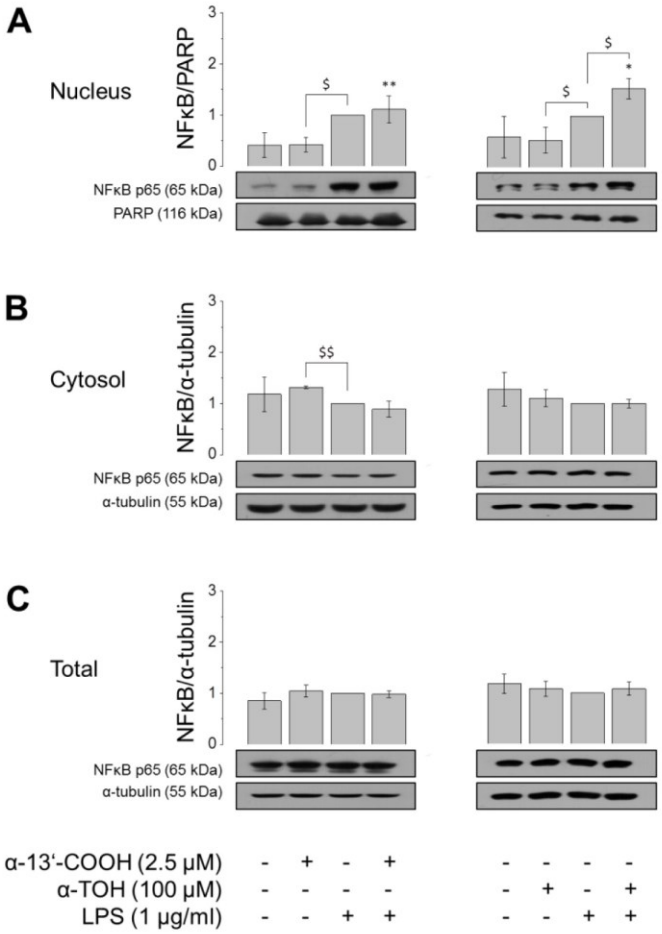

Figure 4: Inhibitory effects by α-13'-COOH are independent from the translocation of the NFκB subunit p65. Murine RAW264.7 macrophages were pre-incubated with solvent, 2.5 µM α-13'-COOH or 100 µM α-TOH for 24 h followed by 1 µg/ml LPS for one further hour. Separated nuclear **(A)** or cytosolic **(B)** fractions and total cell lysates **(C)** were used to analyze translocation of the NFκB p65 subunit. PARP **(A)** and α-tubulin **(B,C)** were used as reference proteins. **(A)** Lipopolysaccharide induced translocation of the p65 subunit from the cytosol into the nucleus which was not blocked by α-13'-COOH (left). Interestingly, p65 translocation was augmented significantly by α-TOH to 154% ± 20% ($p < 0.05$) compared to LPS-stimulated samples (right). **(B)** In the cytosol no changes in p65 subunit levels were observed during co-incubation with LPS and α-13'-COOH or α-TOH compared to LPS-stimulated samples. In the cytosolic fraction levels of the p65 subunit were slightly but

significantly higher after α-13'-COOH incubation by 32% ± 2% ($p < 0.01$) *vs.* stimulation with LPS, whereas α-TOH did not change p65 levels in the cytosolic fraction. Expression of PARP and α-tubulin remained unchanged under all conditions. Representative images of Western blots are shown. Means and standard deviations of three (α-13'-COOH) or four (α-TOH) independent biological experiments are shown. *, $p < 0.05$, **, $p < 0.01$; (*vs.* control); $^{\$}$, $p < 0.05$, $^{\$\$}$, $p < 0.01$ (*vs.* LPS treatment).

3.4 Manuscript IV

First analysis of α-13'-COOH (13'(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid) a long-chain metabolite of vitamin E in human serum

- Methods manuscript -

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Abstract

Despite many years of research, metabolism of vitamin E is not yet fully understood. Within the group of vitamin E α-tocopherol (TOH) is assigned as the most active isomer. Hepatic metabolism of α-TOH is initiated by CYP4F2/CYP3A4-dependent ω-hydroxylation followed by β-oxidation in peroxisomes, which consecutively results in the formation of hydroxychromanol 13'-OH and carboxychromanol 13'-COOH. Further side-chain truncation of these long-chain metabolites (LCM) by β-oxidation cycles forms intermediate-chain (ICM) and short-chain metabolites (SCM), which are subsequently excreted mainly via urine.

In contrast to other vitamin E isomers LCMs of α-TOH have not been found in human serum, yet. We are the first research group able to detect α-13'-COOH in human serum at baseline and after one week supplementation using LC/MS-QTOF. Two different preparation protocols, which differ mainly in enzymatic processing, organic extraction solvents and the used amount of serum, were assessed for the analysis of LCMs. Serum concentrations of α-13'-COOH differ significantly before and after supplementation and between the applied protocols.

The detection of α-13'-COOH in human serum represents an important analytical advancement in the study of vitamin E metabolism in humans and suggests physiological relevance of this LCM.

Keywords

α-tocopherol, LC/MS-QTOF, vitamin E metabolism, physiological LCM

Introduction

Vitamin E isomers (α-, β-, γ-, δ-tocopherol (TOH) and -tocotrienol) differ in characteristic methylation patterns of the hydroxychromanol ring and saturation of the side-chain. Within this group α-TOH is assigned the most active vitamer [1,2], known for its antioxidant and non-antioxidant properties [3]. Despite extensive research over the last decades, knowledge about vitamin E metabolism is incomplete. Hence its widespread use as supplement is questionable.

Hepatic α-TOH is the preferred substrate of α-tocopherol-transfer protein (α-TTP) which facilitates α-TOH transport to non-hepatic tissues by lipoproteins [4–6]. Additionally α-TOH is metabolized by side-chain truncation initiated by CYP4F2/CYP3A4-dependent ω-hydroxylation, which results in formation of hydroxychromanol α-13'-OH (13'-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanol) [7,8]. Subsequent β-oxidation in peroxisomes forms carboxychromanol α-13'-COOH (13'-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid). Further catabolism of these long-chain metabolites of α-TOH (α-LCM) by β-oxidation cycles in peroxisomes and mitochondria results in formation of intermediate-chain (ICM) and short-chain metabolites (SCM), e.g. α-carboxyethylhydroxychromanol (α-CEHC) (Figure 1). The SCMs are commonly found in murine and human urine samples [9,10]. α-Carboxyethylhydroxychromanol as the end-product of the α-TOH catabolism is accepted as a marker for the vitamin E supply in humans [9].

Next to α-TOH, γ-TOH is an important vitamin E isomer in human nutrition and is the second most abundant isomer occurring in human serum [11,12]. The binding specificity of α-TTP is less for γ-TOH [13]; in contrast the specificity to ω-hydroxylation-mediating CYP4F2 is probably higher compared to α-TOH [14]. Therefore the degradation process of γ-TOH and further non-α-TOH isomers is faster than for α-TOH [15]. Due to this physiological serum concentration of γ-TOH is one tenth of α-TOH and decreases further under α-TOH supplementation [16].

Most γ- and δ-metabolites are detectable in fecal samples of rodents and humans and

partially in human plasma [2], but not the metabolites of the α-isomer of vitamin E. The LCMs of α-TOH, α-13'-OH and α-13'-COOH have recently been identified for the first time in human liver cells [8,17], and in human serum by our group [18].

Here we report for the first time an optimized sample preparation for the detection of α-13'-COOH in human serum analyzed by LC/MS-QTOF in comparison to further analytical approaches such as HPLC-ECD and GC/MS. The detection of α-13'-COOH represents an important analytical advancement in the study of vitamin E metabolism in humans and give a first hint for the physiological relevance of this α-LCM.

Materials and methods

Reagents and Material

β-glucuronidase (E. coli Type IX-A), sulfatase (Helix promatia H-1), BHT (butylated hydroxytoluene), dichlormethane and methanol (HPLC-grade) were obtained from Sigma Aldrich (Milan, Italy), Hexane from Panreac (Cinisello Balsamo, Italy), sodium perchlorate monohydrate from Fluka (Milan, Italy) and serum Monovettes® (S-Monovette 9.0 ml Z) from Sarstedt (Nümbrecht, Germany).

Human study and serum sample collection

For the pilot study healthy male and female, non-smoking, normolipemic volunteers with no medications known to affect xenobiotic metabolism declared written consent to participate in one week supplementation of 1000 IU *RRR*-α-TOH/d and to further blood withdrawal as well as analysis of their serum samples. Supplementation was carried out using one soft gel capsule containing 1000 IU (671.37 mg) *RRR*-α-TOH, soybean oil and sorbitol (Optovit®, Hermes Arzneimittel GmbH) at breakfast time with a regular meal consisting of milk, bread, marmalade, cheese or ham. Dietary intake of vitamin E was maintained the same during the protocol and was assumed to be negligible with respect to the supplemented vitamin E dosis. Blood sample collection was carried out of fasted volunteers at baseline and on day eight of the supplementation protocol. Samples were prepared using serum Monovettes® (Sarstedt, Nümbrecht, Germany) by centrifugation (2000 × g) of blood specimens for 10 min at 4°C. Subsequently samples were stored at -80°C until analysis.

Extraction procedure

Two different extraction protocols were assessed for the analysis of α-LCMs (Figure 2). For protocol 1 (M1) 25 µl of 10 mg/ml ascorbic acid, 1500 IU of *E. coli* β-glucuronidaseType IX-A and 26 IU/ml sulfatase (*Helix promatia H-1*) were added to serum (500 µl) in glass tubes. Both of the enzymes were prepared in 200 µl of 0.25 M sodium acetate puffer (pH 6). After mixing gently the samples were incubated for 30 min at 34°C under N_2 atmosphere.

Extraction was performed twice using 5 ml hexane/dichlormethane (5/2 v/v) containing 1% butylated hydroxytoluene (BHT) (w/v). After mixing for 1 min at room temperature, the samples were centrifuged (2000 × g, 15 min, 10°C) and the upper organic layer was collected in glass tubes. The aqueous phase obtained with this procedure can be used for further extraction of SCMs as described in Freiser & Jiang, which was not the aim of this study.

Organic fractions were dried down under a stream of N_2 and then resuspended in 50 µl MeOH for LC/MS-QTOF analysis or 50 µl mobile phase (80% MeOH, 20% ultra-pure water, 10 mM sodium perchlorate monohydrate) for HPLC-ECD analysis.

In the second protocol (M2), among other differences, LCM extraction was carried out without enzymatic processing of conjugated forms. 4 ml of serum were mixed in a glass tube with 80 mg ascorbic acid before incubation for 5 min at 60°C in a water bath. Following 150 µl of 12 M HCl were added and serum was incubated for 1 h at 60°C in heated water bath under N_2 atmosphere. Subsequently three extraction steps were performed with 8, 6 and 4 ml hexane, respectively. Samples were centrifuged at 2000 × g (4°C and 10 min) after each extraction and the supernatant was collected and was partially evaporated by a stream of N_2 until 1 ml of residual organic solvent was attained. This was transferred to a 2 ml glass vial to complete the evaporation protocol and subsequently the sample was dissolved in 50 µl MeOH (LC/MS-QTOF) or 50 µl of the HPLC-ECD mobile phase (80% MeOH, 20% ultra-pure water, 10 mM sodium perchlorate monohydrate).

Plasma analyses

Analytical procedures used for this study included GC/MS, HPLC-ECD and LC/MS-QTOF.

GC/MS

Standard of the LCM α-13'-COOH was derivatized with pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). After 1 h incubation at 70°C and drying under N_2, standard was dissolved in methanol. For the analysis of the metabolites Gas Chromatography-System 7890A coupled with 5975C VL MSD with Triple-Axis Detector from

Agilent Technologies was used. The used carrier gas was helium. The separation of the compounds was achieved using a HP-5 cross-linked methylsioxane column with dimensions 30 m x 0.25 mm i.d. and film thickness 0.25 x m. The oven temperature was 50°C followed by an increase of 50°C/min to 310°C and was maintained for 15 min [19]. The injection volume was 1 µl. The transfer line and the MS source temperature were at 290°C and 200°C, respectively. In selected ion mode the *m/z 604.4* corresponding to the molecular ion of α-13'-COOH was monitored. In parallel we measured α-TOH and γ-TOH. As expected α-TOH increased after supplementation, whereas γ-TOH decreased partly up to not detectable levels during supplementation (data not shown).

LC/MS-QTOF

Sample analyses were performed on an Agilent 6540 ultra high definition (UHD) Accurate-Mass Q-TOF LC/MS system (Agilent, Palo Alto, CA, USA) equipped with an Agilent Dual Jet Stream Technology ESI source operating in positive and negative ion mode (fragmentor 300 V). The chromatographic separation was achieved with Zorbax Eclipse Plus C18 column (2.1x50 mm, 1.8 µm) maintained at 35°C at flow rate of 0.2 ml/min with a binary gradient of solvent A (H_2O including 0.1% formic acid) and B (MeOH). The gradient was (i) 0 min 80%, (ii) 3 min up to 99% (iii) and finally 15 min with 99% of solvent B. The injection volume was 3 µL. Spectrometric data were recorded in the range of 100-1000 m/z at 2 GHz. The MS/MS spectrum was obtained with a collisional energy of 40 V with N_2. All LC/MS-QTOF system was governed via the Agilent MassHunter Workstation software [18].

Analyses of α-TOH metabolite α-13'-COOH were performed by both positive and negative acquisition method and total ion count (TIC)-mode with $[M-H]^-$ deprotonated molecules (*m/z 459.4*) as well as $[M+H]^+$ protonated molecules (*m/z 461.4*). Dependent of the molecule structure α-TOH was measured in positive acquisition method and TIC-mode with $[M+H]^+$ protonated molecules (*m/z 431.4*). Used mobile phase contained 80% methanol, 20% ultra-pure H_2O with 0.1% acetyl fluor (AcF). For the calibration external standard curves of α-TOH and α-13'-COOH were performed.

HPLC-ECD

Samples were quantified by HPLC-ECD using a glassy carbon cell with working electrode set at 750 mV oxidation potential against a AgCl reference electrode (EG&G Princeton Applied Research, model 400, EC Detector). A reversed-phase Kromasil C8 column (5 µm, 250 x 4.6 mm i.d.) was used as stationary phase protected with a guard pre-column containing the same phase. Mobile phase was 90% methanol and 10% ultra-pure H_2O containing 10 mM sodium perchlorate monohydrate. Separation was carried out under isocratic conditions at a flow rate of 1.0 ml/min. 10 µl and 50 µl samples were injected to quantify TOHs and metabolites, respectively. Chromatographic data were analyzed with JASCO-BORWIN Chromatography software version 1.50. For the calibration external standard curves for α-TOH, γ-TOH and α-13'-COOH were performed, respectively. The detection limit is 10 nM to 20 nM.

Results

GC/MS

Using the GC/MS analysis protocol described in the section “Materials and Methods”, neither the main ion nor ionization products of the derivatized LCM α-13’-COOH were detected (not shown). The size of the silylated metabolite and its poor fragmentation pattern upon electron ionization could explain this negative result.

HPLC-ECD

The separation of α-TOH by HPLC and subsequent electrochemical detection was successful using mobile phase containing 90% methanol, 10% ultra-pure H_2O and 10 mM sodium perchlorate monohydrate (data not shown). Based on its chemical structure α-13’-COOH retention time is shorter compared to α-TOH. Despite optimization of the injection volume and mobile phase α-13’-COOH could not be detected clearly as a single distinct peak in used serum samples.

α-13’-COOH in human serum detectable by LC/MS-QTOF

Birringer as well as Zhao and colleges were able to detect the LCM α-13’-COOH in human liver cells and mice fecal samples, respectively [7,8]. Additionally many LCMs, ICMs and SCMs have been identified in human serum [2]. However the identification of α-13’-COOH in human serum is still missing. α-13’-COOH and its metabolic precursor α-TOH were measured by LC/MS-QTOF. We detected successfully both compounds in un-supplemented (Figure 3) and post-supplemented serum samples (see Table 1). The retention times were 3.04 min to 3.06 min for α-13’-COOH and 4.18 min for α-TOH. The concentration of α-TOH increased after supplementation but varies between the donors from 2-7 fold compared to control samples (see Table 1). Higher concentrations of α-TOH cause an increase in the associated α-TOH-metabolism including the formation of α-13’-COOH. Using our optimized sample preparation protocol (M1) measured with negative acquisition method we detected α-13’-COOH at baseline concentration of 13.2 nM (donor 1) and 12.5 nM (donor 2). The

concentration of α-13'-COOH in supplemented samples increased for donor 1 and 2 up to 4.6 and 4.4 fold, respectively, compared to un-supplemented samples taken from fasting volunteers.

Extraction solvents, enzymatic processing and used acquisition method are responsible for the α-13'-COOH recovery

Two different extraction protocols were performed (see "Material and Methods"). The hexane/dichlormethane/BHT-extraction solvent in combination with treatment with β-glucuronidase and sulfatase (M1) results in a higher recovery of α-13'-COOH in serum taken from un-supplemented and supplemented volunteers (Table 1B) compared to method 2 (M2) (Table 2). Method 2 showed equal amounts of α-13'-COOH in each sample, whereas measurements of M1 processed samples showed (i) higher basal levels 13.2 nM and 12.5 nM as well as (ii) increased concentrations in serum samples after supplementation 60.3 nM (donor 1) and 54.7 nM (donor 2) (Table 1B). Normalization to an equal amount of used extracted serum (0.5 ml) quantified the difference in concentration of α-13'-COOH detected between M1 and M2 in un-supplemented and supplemented samples as 10 fold and 40 fold, respectively (Table 2). The recovery of α-TOH in serum samples is comparable between both protocols (data not shown).

For further optimization of the LC/MS-QTOF measurement negative acquisition method was compared to positive acquisition method, as used for α-TOH. In result α-13'-COOH concentration detected by positive acquisition method was two to three fold more efficient in supplemented samples compared to negative acquisition method (Table 1B).

Discussion

Findings in cell models and rodent *in vivo* studies of the last 20 years help to understand and extend the metabolite profile of vitamin E metabolism, but it is not complete, yet. Vitamin E as a group of multi-structural compounds passes isomer-specific intermediates and reaches variable concentrations in different tissues and body fluids [20]. Mainly the γ- and δ-LCMs, ICMs and SCMs have been detected in human cell line A549, rat liver, human as well as rodent serum, feces and urine [19,21,22]. Birringer *et. al* recently showed the metabolism of TOHs to their LCMs in HepG2 cells, detected by HPLC/UV [17]. Additionally the LCMs of α-TOH, α-13'-OH and α-13'-COOH, have recently been identified in feces [19], but not yet in human plasma. The major finding of this study is the identification of α-13'-COOH in human serum even under un-supplemented conditions by LC/MS-QTOF. The physiological concentration of α-TOH in plasma of healthy, un-supplemented volunteers is up to 25 µM. Our analyzed serum contains 6 µM ± 1 µM. As previously described the serum concentration of α-TOH increases after supplementation with *RRR*-α-TOH [23], in our case by an average of 2 to 7 fold, depending on the donor. Irrespective of the increasing effect due to supplementation, significant differences in α-13'-COOH serum concentration were found using two different protocols, see "Materials and Methods". Despite starting with less serum in method 1 (M1) the yield of α-13'-COOH after this extraction is 10 fold and 40 fold higher in baseline and supplemented samples, respectively, in comparison to method 2 (M2). The reason for this result could be the enzymatic processing in M1 using β-glucuronidase and sulfatase. As already known, carboxychromanols, such as α-13'-COOH usually occur in human cells and rats in conjugated forms (sulfate or phosphate) [19,24]. Freiser *et al.* described detection of different metabolites before and after enzymatic processing. On the other hand the different polarities of used organic extraction solvents may be responsible for the different metabolite yield in our methods, further experiments are necessary to decide with certainty. In addition we found optimal measurement conditions for α-TOH and α-13'-COOH using positive acquisition method of LC/MS-QTOF instead of negative acquisition method. This could possibly be explained by the structural composition of the vitamin E compounds.

Apart from the detection of α-13'-COOH via LC/MS-QTOF, recently described methods to analyze TOHs and their metabolites such as GC/MS and HPLC-ECD were used. The reason for not detecting this metabolite by these methods could be its size after the silylation process or its poor fragmentation response upon electron ionization in the GC/MS assay. As recently described 13'-COOH is detectable by fluorescence HPLC [17,19,24]. We used HPLC with an electrochemical detector which is characterized as a highly sensitive method used to detect compounds up to nanomolar amounts. The retention time of our metabolite of interest, α-13'-COOH, could be close to that of tocotrienols and many other compounds in human serum. This could be the reason for not detecting a unique single peak for α-13'-COOH in our samples under the current chromatographic conditions. As mobile phase 90% methanol, 10% ultra-pure H_2O and 10 mM sodium perchlorate monohydrate was used initially. To optimize the separation within the first minutes of the HPLC analysis reduction methanol content to 80% and 70% was tested without achieving a satisfactory result. For further investigations of metabolites in human serum previous separation of compound of interest via preparative HPLC or a different approach to remove disturbing compounds in serum is useful [24].

With this study we described for the first time an optimized sample preparation and detection method by LC/MS-QTOF for the recently published detection of the physiological LCM α-13'-COOH in human serum by our group [18]. This metabolite was detectable at baseline serum level and increased after one week supplementation with *RRR*-α-TOH. The aim of further studies is to clarify the pharmacokinetic of this metabolite and to understand the possible influence of individual parameters such as gender, age or life style (smoking, drugs), which are known to modulate Vitamin E metabolism [25].

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References

[1] J. Tucker, D. Townsend, Alpha-tocopherol: roles in prevention and therapy of human disease, Biomed Pharmacother 59 (2005) 380-387.

[2] Y. Zhao, M.-J. Lee, C. Cheung, J.-H. Ju, Y.-K. Chen, B. Liu, L.-Q. Hu, C.S. Yang, Analysis of Multiple Metabolites of Tocopherols and Tocotrienols in Mice and Humans, J Agric Food Chem 58 (2010) 4844-4852.

[3] A. Azzi, R. Gysin, P. Kempná, A. Munteanu, Y. Negis, L. Villacorta, T. Visarius, J.-M. Zingg, Vitamin E mediates cell signaling and regulation of gene expression, Ann N Y Acad Sci 1031 (2004) 86-95.

[4] M.G. Traber, H.J. Kayden, Preferential incorporation of alpha-tocopherol vs gamma-tocopherol in human lipoproteins, Am J Clin Nutr 49 (1989) 517-526.

[5] H. Weiser, G. Riss, A.W. Kormann, Selective biodiscrimination of alpha-tocopherol stereoisomers. Similar enrichment of all 2R forms in rat tissues after oral all-rac-alpha-tocopheryl acetate, Ann N Y Acad Sci 669 (1992) 393-395.

[6] C. Kiyose, R. Muramatsu, Y. Kameyama, T. Ueda, O. Igarashi, Biodiscrimination of alpha-tocopherol stereoisomers in humans after oral administration, Am J Clin Nutr 65 (1997) 785-789.

[7] M. Birringer, D. Drogan, R. Brigelius-Flohé, Tocopherols are metabolized in HepG2 cells by side chain omega-oxidation and consecutive beta-oxidation, FRBM 31 (2001) 226-232.

[8] D.J. Mustacich, S.W. Leonard, N.K. Patel, M.G. Traber, Alpha-tocopherol beta-oxidation localized to rat liver mitochondria, FRBM 48 (2010) 73-81.

[9] K.M. Lebold, A. Ang, M.G. Traber, L. Arab, Urinary α-carboxyethyl hydroxychroman can be used as a predictor of α-tocopherol adequacy, as demonstrated in the Energetics Study, Am J Clin Nutr 96 (2012) 801-809.

[10] J.-Y. Cho, D.W. Kang, X. Ma, S.-H. Ahn, K.W. Krausz, H. Luecke, J.R. Idle, F.J. Gonzalez, Metabolomics reveals a novel vitamin E metabolite and attenuated vitamin E metabolism upon PXR activation, J Lipid Res 50 (2009) 924-937.

[11] E. Cardenas, R. Ghosh, Vitamin E: A dark horse at the crossroad of cancer management, Biochem Pharmacol 86 (2013) 845-852.

[12] W.A. Behrens, R. Madère, Alpha- and gamma tocopherol concentrations in human serum, J Am Coll Nutr 5 (1986) 91-96.

[13] A. Hosomi, M. Arita, Y. Sato, C. Kiyose, T. Ueda, O. Igarashi, H. Arai, K. Inoue, Affinity for alpha-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs, FEBS Lett 409 (1997) 105-108.

[14] T.J. Sontag, R.S. Parker, Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status, J Biol Chem 277 (2002) 25290-25296.

[15] N. Grebenstein, M. Schumacher, L. Graeve, J. Frank, α-Tocopherol transfer protein is not required for the discrimination against γ-tocopherol in vivo but protects it from side-chain degradation in vitro, Mol Nutr Food Res (2014) n/a.

[16] C. Kiyose, H. Saito, K. Kaneko, K. Hamamura, M. Tomioka, T. Ueda, O. Igarashi, Alpha-tocopherol affects the urinary and biliary excretion of 2,7,8-trimethyl-2 (2'-carboxyethyl)-6-hydroxychroman, gamma-tocopherol metabolite, in rats, Lipids 36 (2001) 467-472.

[17] M. Birringer, D. Lington, S. Vertuani, S. Manfredini, D. Scharlau, M. Glei, M. Ristow, Proapoptotic effects of long-chain vitamin E metabolites in HepG2 cells are mediated by oxidative stress, FRBM 49 (2010) 1315-1322.

[18] M. Wallert, S. Mosig, K. Rennert, H. Funke, M. Ristow, R.M. Pellegrino, G. Cruciani, F. Galli, S. Lorkowski, M. Birringer, Long-chain metabolites of α-tocopherol occur in human serum and inhibit macrophage foam cell formation in vitro, FRBM 68 (2013 // 2014) 43-51.

[19] Q. Jiang, H. Freiser, K.V. Wood, X. Yin, Identification and quantitation of novel vitamin E metabolites, sulfated long-chain carboxychromanols, in human A549 cells and in rats, J Lipid Res 48 (2007) 1221-1230.

[20] M. Birringer, P. Pfluger, D. Kluth, N. Landes, R. Brigelius-Flohé, Identities and differences in the metabolism of tocotrienols and tocopherols in HepG2 cells, J Nutr 132 (2002) 3113-3118.

[21] H. Freiser, Q. Jiang, Gamma-tocotrienol and gamma-tocopherol are primarily metabolized to conjugated 2-(beta-carboxyethyl)-6-hydroxy-2,7,8-trimethylchroman and sulfated long-chain carboxychromanols in rats, J Nutr 139 (2009) 884-889.

[22] F. Galli, R. Lee, C. Dunster, F.J. Kelly, Gas chromatography mass spectrometry analysis of carboxyethyl-hydroxychroman metabolites of alpha- and gamma-tocopherol in human plasma, FRBM 32 (2002) 333-340.

[23] M. Schultz, M. Leist, M. Petrzika, B. Gassmann, R. Brigelius-Flohé, Novel urinary metabolite of alpha-tocopherol, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman, as an indicator of an adequate vitamin E supply?, Am J Clin Nutr 62 (1995) 1527S-1534S.

[24] H. Freiser, Q. Jiang, Optimization of the enzymatic hydrolysis and analysis of plasma conjugated gamma-CEHC and sulfated long-chain carboxychromanols, metabolites of vitamin E, Anal Biochem 388 (2009) 260-265.

[25] C.H. Johnson, O. Slanar, K.W. Krausz, D.W. Kang, A.D. Patterson, J.-H. Kim, H. Luecke, F.J. Gonzalez, J.R. Idle, Novel metabolites and roles for -tocopherol in humans and mice discovered by mass spectrometry-based metabolomics, Am J Clin Nutr 96 (2012) 818-830.

Table legends

Table 1

Detection of (A) α-TOH and (B) α-13-COOH by LC/MS-QTOF after extraction method 1 (M1) measured in negative and positive acquisition method. Healthy volunteers were supplemented with 1000 IU/d α-TOH for one week. Before and after supplementation blood samples were taken to gain serum for metabolite analysis by LC/MS-QTOF. **(A)** α-TOH was measured by positive acquisition method, whereas **(B)** α-13'-COOH was measured with both positive and negative acquisition method. Concentrations of α-TOH and α-13'-COOH increased after one week supplementation with 1000 IU/d *RRR*-α-TOH. Differences in α-TOH varied between donor 1 and 2 from two to seven fold compared to un-supplemented samples. Concentrations of α-13'-COOH are more consistent between both donors at baseline (13.2 nM and 12.5 nM) and after supplementation (60.3 nM and 54.7 nM). **(B)** Change to positive acquisition method increased the detected concentration of α-13'-COOH two to three fold compared to the negative acquisition method.

(A)

	Positive acquisition method
Samples	α-TOH [µM] (M1)
Donor 1 control	6.6
Donor 1 supplemented	11.4
Donor 2 control	4.9
Donor 2 supplemented	34.4

(B)

	Negative acquisition method	*Positive* acquisition method
Samples	α-13'-COOH [nM] (M1)	α-13'-COOH [nM] (M1)
Donor 1 control	13.2	
Donor 1 supplemented	60.3	120.2
Donor 2 control	12.5	
Donor 2 supplemented	54.7	172.7

Table 2

Extraction solvents and application of β-glucuronidase and sulfatase are essential for α-13'-COOH recovery. Normalization to an equal amount of serum (0.5 ml) showed differences in concentration of detected α-13'-COOH using extraction method 1 (M1) including β-glucuronidase and sulfatase treatment instead of method 2 (M2). Recovery of α-13'-COOH by using method 1 was ten fold higher for the control and even 40 fold in supplemented samples.

Negative acquisition method		
Samples	α-13'-COOH [nM] (M1)	α-13'-COOH [nM] (M2)
Donor 1 control	13.2	1.3
Donor 1 supplemented	60.3	1.5
Donor 2 control	12.5	1.3
Donor 2 supplemented	54.7	1.4

Figure legends

Figure 1: Vitamin E metabolism in humans. Within the group of vitamin E α-TOH is assigned as the most important vitamer, therefore the metabolism shown here focuses on degradation of α-TOH. In the liver vitamin E metabolism occurs in three compartments: microsomes, peroxisomes and mitochondria. In microsomes the long-chain metabolite (LCM) α-13'-OH is formed by CYP4F2/CYP3A4-dependent ω-hydroxylation followed by α-oxidation which result in formation of α-13'-COOH. Subsequent two β-oxidation steps in peroxisomes and three β-oxidation steps in mitochondria form intermediates (ICM) and finally short-chain metabolites (SCM) which are mainly excreted via urine.

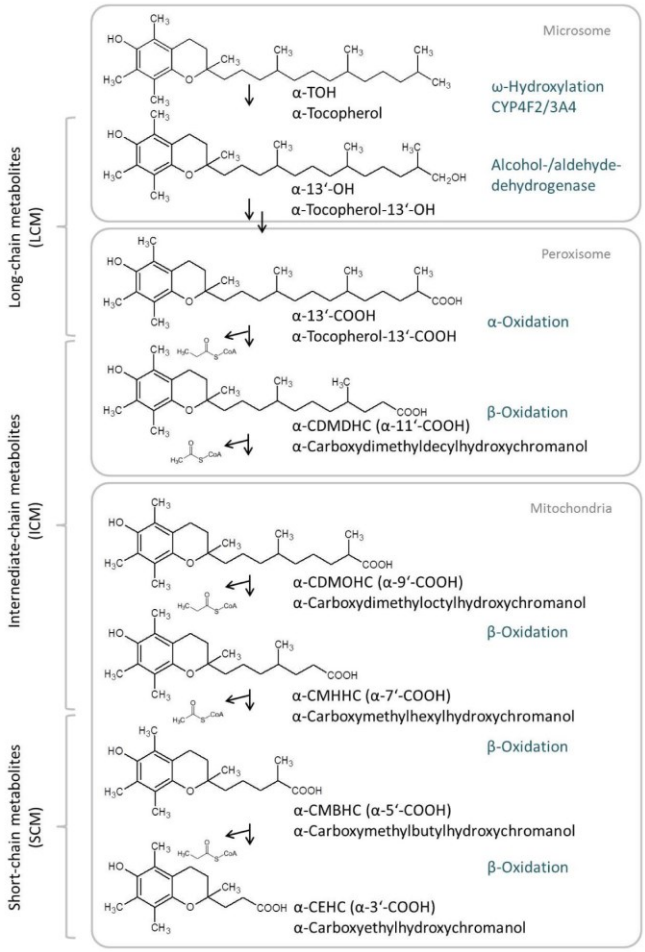


Figure 2: Comparison of extraction method (A) 1 and (B) 2. The two extraction methods differ in used amount of serum **(A)** 0.5 ml and **(B)** 4 ml, enzymatic treatment **(A)** β-glucuronidase and sulfatase and **(B)** none, incubation conditions and extraction solvents **(A)** hexane/dichlormethane including BHT and **(B)** hexane.

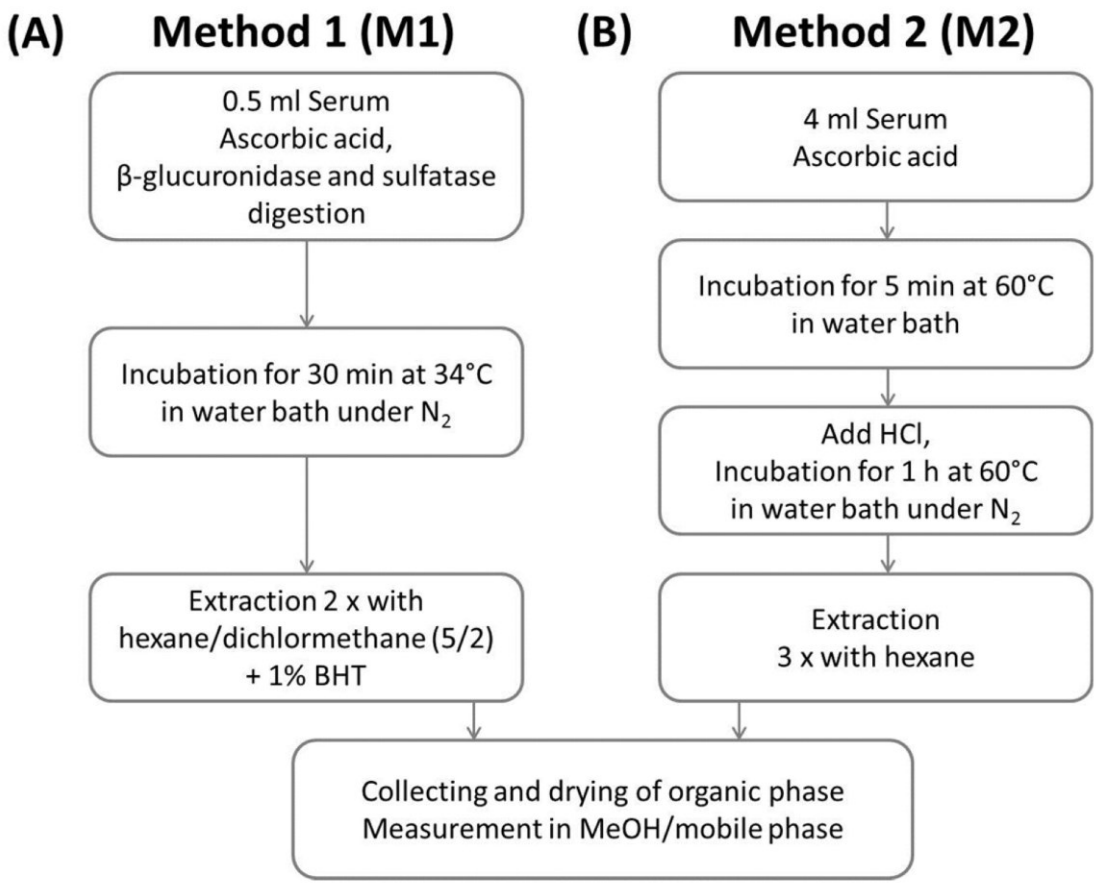
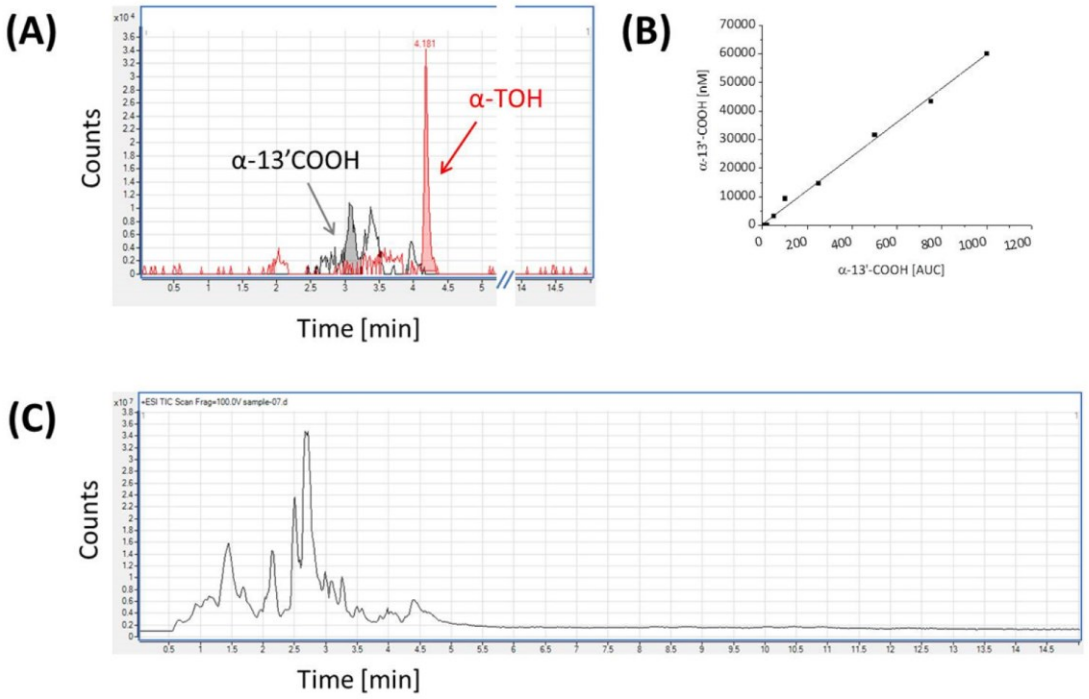


Figure 3:

Detection of α-13'-COOH in human serum using LC/MS-QTOF in positive acquisition method. Overlay of the chromatograms of α-13'-COOH and α-TOH in serum obtained from a female volunteer. **(A)** The metabolite α-13'-COOH was analyzed in human un-supplemented serum samples by positive acquisition method and TIC mode with $[M+H]^+$ protonated molecules (*m/z* 461.4). **(B)** Additionally the standard curves of α-13'-COOH and the **(C)** full mass chromatogram of the serum analyzed in TIC mode are shown.



4 Discussion

To say it with Eduardo Cardenas´ words: "This is a very exciting time in vitamin E research, yet it is evident that we are far away from making the final decision on the benefit vs. risk for the potential use of vitamin E in human health" (CARDENAS & GHOSH 2013). The following points will give an insight into current problems and obscure or even questionable claims about vitamin E to understand its physiological mode of action, its metabolism and the discrepancies between *in vivo* and *in vitro* results.

4.1 Individual differences in vitamin E utilization and metabolism

Several years ago vitamin E was characterized as an *in vitro* and *in vivo* radical chain-breaking antioxidant (BURTON & INGOLD 1989). Despite intensive investigations over decades vitamin E metabolism and antioxidant dependent and independent properties have not yet been fully understood. Nevertheless, vitamin E is widely used as a nutritional supplement (TRABER 2004) to prevent age-related diseases such as cardiovascular outcomes, which is probably not entirely without side-effects. Although *in vitro* data and animal trials have shown evidence for antiatherogenic effects, confirmation in clinical trials failed (LIBINAKI *et al.* 2010; WALLERT *et al.* 2014). Since meta-analyses revealed a lack of protection against cardiovascular events, a profound understanding of vitamin E metabolism and its biological activity is mandatory. Possible explanation can be the difference in dose-effect and also in the form of the applied vitamin E (natural or synthetic), e.g. *RRR*-α-TOH or stereoisomer mixture (KEANEY *et al.* 1994; LIBINAKI *et al.* 2010).

For our own *in vivo* studies we used the natural form *RRR*-α-TOH for supplementation. After one week of supplementation with 1000 IU/d α-TOH serum levels have increased. Additionally we detected the LCM of α-TOH, α-13'-COOH, in serum at baseline and in higher amounts after supplementation. Next to differences due to the methodical procedure we observed interindividual differences in the amount of both α-TOH and α-13'-COOH levels. Discrepancies of vitamin E-related outcomes of human *in vivo* studies may be caused by individual differences in intestinal absorption efficiency. As known for drugs and other compounds such as β-carotin so-called non-, low or high responder exist (BOREL *et al.* 1998; BROWN *et al.* 1989; CHEON *et al.* 2006). Non-responders will likely not benefit from the suspected antioxidant properties of vitamin E, thus they could be more susceptible than responders to diseases in which free radicals are involved (BOREL *et al.* 1998). Since vitamin E has the highest turnover rate in the liver (BURTON & INGOLD 1989) it may be possible that high responders in contrast to "normal" responders tend to degrade vitamin E faster and even more and therefore benefit less from vitamin E mediated effects. This could be the reason for differences in human supplementation studies. Whereas in non-responders supplementation with *RRR*-α-TOH do not have any effect high-responders may profit from

supplementation.

Since we assume that the mode of action of vitamin E is mediated by the formation of the LCMs, intervention studies with high supplementation of vitamin E possibly should show protective effects. A closer look to primary and secondary clinical prevention trials of high doses of vitamin E (600 IU - 800 IU) supplementation did not reveal clear results in correlation to CVD. The large-scaled Woman's Health Study (WHS) and the Woman's Antioxidant Cardiovascular Study (WACS) for example only showed invers association in secondary outcomes. Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE) and Cambridge Heart Antioxidant Study (CHAOS) showed evidence for an inverse correlation between vitamin E supplementation and CVD. In contrast the HDL-Atherosclerosis Treatment Study (HATS) and a large-scaled study from the Medical Research Council/British Heart Foundation (MRC/BHF) did not show any effect of natural or synthetic vitamin E supplementation. In contrast supplementation of 3200 IU *RRR*-α-TOH/d for 20 weeks suppressed plasma F_2-isoprostane, a marker for lipid peroxidation, in participants with increased CVD risk (ROBERTS *et al.* 2007). Hence a more detailed analysis of the LCMs formed during vitamin E supplementation in those and further studies would be of interest to verify the contribution of the metabolites to vitamin E effects.

On the other hand the metabolites` biological activity and function is distinct from their precursor and could be of interest and will be therefore discussed in the following chapters. Vitamin E is the highest accumulated lipid soluble vitamin in plasma and LDL (TRABER 2007). Thereby its transport to extra-hepatic tissues is efficient and is the basis for its relevance for biological properties such as antioxidant and antiinflammatory effects (LIBINAKI *et al.* 2010). Additionally vitamin E import, export and distribution are regulated by several receptors and proteins. Their individual activities in humans are important as been shown for α-TTP deficiency in AVED patients which leads to impaired distribution deficiency of α-TOH (CARDENAS & GHOSH 2013; TRABER 2007). In these cases it is quite reasonable to supplement vitamin E by taking into account the individual therapeutic window (LIBINAKI *et al.* 2010). Based on this it is possible that either absorption or metabolic polymorphisms may exist in humans which influence vitamin E metabolism and activity (BOREL *et al.* 1998). By the metabolism of vitamin E hydrophilic metabolites which are called LCMs are formed mainly from non-α-TOH isomers. The formed SCMs are excreted via the urine possibly because non-α-TOH isomers are less active as antioxidants compared to α-TOH (TRABER 2013). The excessive intake of vitamin E elevates its own hepatic uptake via SR-B1 (MUSTACICH *et al.* 2009) and its subsequent metabolism. The aim of the enhanced degradation of vitamin E is likely the protection against toxic accumulation in human organs in contrast to the fat-soluble vitamins A and D.

4.1.1 Interaction of vitamin E metabolism with xenobiotic detoxification

The pathway of vitamin E catabolism is in part similar to xenobiotic detoxification. Hepatic xenobiotic metabolism occurs in three phases: (I) oxidation, (II) conjugation and (III) excretion (TRABER *et al.* 2011). Each metabolic step involves a subset of hepatic proteins such as members of the cytochrome P450 protein family (phase I), sulfotransferases (SULT) and glutathione S-transferases (GST) (phase II), and ABC transporters (phase III) (Figure 13). In mice these hepatic proteins are upregulated by elevated α-TOH intake (MUSTACICH *et al.* 2009; TRABER 2008). Among the phase III transporter systems regulating excretion of xenobiotic compounds and their metabolites from the liver the MDRs, such as the xenobiotic export transporter MDR1, is of particular importance within the ABC superfamily (MUSTACICH *et al.* 2009; TRABER *et al.* 2011). Based upon several studies on vitamin E metabolism Maret Traber concluded that α-TOH is recognized as a vitamin whereas non-α-TOH isomers are recognized as xenobiotics which are further metabolized to prepare them for elimination (TRABER 2013). Nevertheless our investigations failed to reveal non-α-TOH metabolites in human serum. Although non-α-TOH isomers are known to be metabolized

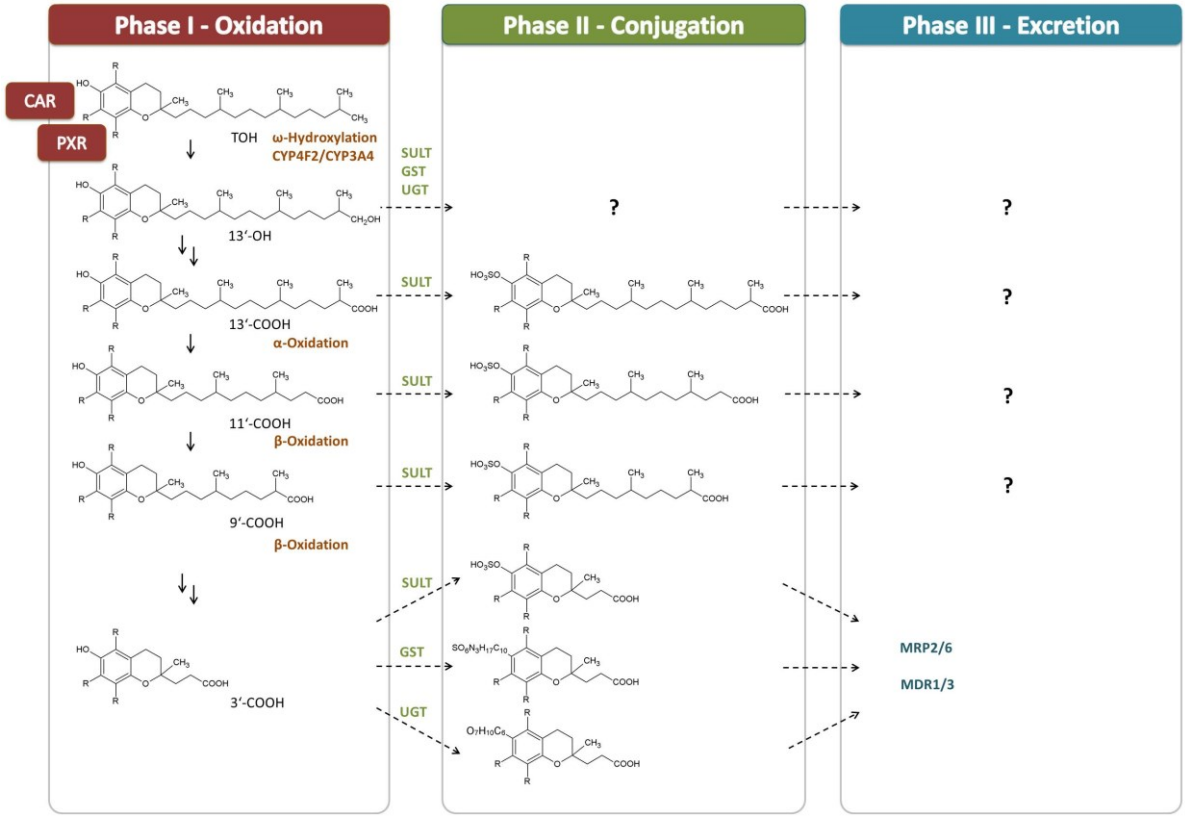


Figure 13: Involvement of xenobiotic-associated pathways and enzymes in vitamin E metabolism, conjugation and excretion. Metabolism of vitamin E in the liver follows the xenobiotic detoxification pathway. First metabolites of vitamin E are formed by CYP3A4- and CYP4F2-dependent ω-hydroxylation and further oxidation cycles similar to xenobiotics. Subsequently conjugates are formed via sulfation (SULT), glucuronidation (UGT) or with glutathione (GST) to facilitate excretion of the vitamin E metabolites and the catabolic end-products in parallel with xenobiotics via multidrug resistance-related proteins (MRP) and MDR (JIANG *et al.* 2007; MUSTACICH *et al.* 2009; TRABER 2010).

fast we only detected α-13'-COOH. The LCMs of δ-TOH, δ-13'-COOH and δ-13'-OH, have not been found in human plasma as well as α-13'-OH, the precursor of α-13'-COOH, possibly because of fast metabolic degradation or insufficient analytics. Since no standards for γ- and β-LCMs are available at present no statement on the existence or accumulation of these metabolites in our samplings can be made. Xenobiotic transporters are candidates to be involved in the regulation of hepatic TOH concentration and CEHC excretion (TRABER *et al.* 2011). One key nuclear receptor in xenobiotic metabolism is among others the PXR (TRABER 2008). It has been proposed that vitamin E interacts with xenobiotic metabolism via binding to the PXR and subsequent formation of the vitamin E PXR/retinoid X receptor (RXR) heterodimer which induces metabolism of vitamin E (LANDES *et al.* 2003; TRABER 2004; TRABER *et al.* 2011). Reports on the regulation of PXR- or CYP3A-dependent metabolism by the α-TOH concentration in the liver are inconsistent and therefore the contribution to the protection from vitamin E excess needs further investigations. An additional hint for the interaction of vitamin E and xenobiotic metabolism is the conjugation, such as sulfation, of intermediate and end-products of the vitamin E catabolism as it is known for xenobiotics (TRABER 2004; TRABER *et al.* 2011) (Figure 13). The conjugation of intermediate metabolites and catabolic products may be important for intracellular trafficking and trafficking from the liver possibly via ABCG2, a transporter responsible for sulfated and glucuronidated compounds export (TRABER *et al.* 2011).

4.1.2 Interaction of the metabolism of vitamin E and K

Since hepatic CYP4F2 is important for the initial step of vitamin E metabolism it is named α-TOH hydroxylase. In addition to vitamin E, CYP4F2 processes long-chain and very long-chain fatty acids such as behenic acid (C22:0), lignoceric acid (C24:0) and cerotic acid (C26:0) (HARDWICK 2008), oleic acid (C18:1), stearic acid (C18:0) and palmitic acid (C16:0) (HSU *et al.* 2007), and inflammatory response-related (HARDWICK 2008) arachidonic acid conversion to 20-hydroxyeicosatetraenoic acid (HETE) (LASKER *et al.* 2000) and leukotriene B_4 (LTB_4) (HSU *et al.* 2007; KIKUTA *et al.* 2000). Cytochrome 4F2-mediated hydroxylation is inducible by the sterol regulatory element-binding proteins (SREBP) and generates bioactive metabolites which act as lipid mediators of signal transduction (HSU *et al.* 2007; KALSOTRA & STROBEL 2006). Parallels also exist between the metabolic pathways of vitamin E and vitamin K (TRABER 2010), e.g. side chain ω-hydroxylation by CYP4F2 and the following β-oxidation. Further PXR binds vitamin E and vitamin K and activates genes involved in xenobiotic detoxification such as CYP3A4 and MDR1 (LANDES *et al.* 2003; LEHMANN *et al.* 1998; MASUYAMA *et al.* 2005; TRABER 2008, 2010). Additionally PXR binds the vitamin K metabolite menaquinone-4. The PXR/menaquinone-4 complex regulates extracellular matrix and collagen formation in osteoblasts (ICHIKAWA *et al.* 2006). Since vitamin K is important for blood coagulation interactions of these vitamins may result in limitation of the active vitamin K form by excessive vitamin E which may explain the association of excessive vitamin E and coagulation disturbances. Therefore vitamin E has been suggested for therapeutic use in patients with increased risk of thrombosis (TRABER 2010).

4.1.3 Further metabolic pathway interactions

As already mentioned in Chapter 1.1.3 parallel intake of α-TOH and other fat-soluble micro-constituents such as γ-TOH and lutein decrease the absorption of α-TOH by more than 40% (γ-TOH) and 20% (lutein). Whereas no significant effects of other carotenoids, such as β-carotene and lycopene, on the absorption of α-TOH have been observed *in vitro* (REBOUL *et al.* 2006). In the case of retinoic acid it has been reported that it inhibits absorption of α-TOH by more than 30% in rats (BIERI *et al.* 1981). As α-TOH is the most active isomer, within the group of vitamin E, its optimal absorption under physiological conditions is important. Inhibitory effects on the absorption of α-TOH, for example from food containing high amounts of inhibitory compounds, have to be considered in the evaluation of individual variations in the metabolism of α-TOH. Next to variations in absorption subsequent transport of vitamin E and its metabolites is a potential source of individual differences. Until now no binding proteins in plasma are known for α-TOH and certainly not for the LCM (BJØRNEBOE *et al.* 1990; TRABER 2013).

4.1.4 Chemical modifications of vitamin E metabolites

Since the enzymes involved in the metabolism of vitamin E play also a role in several other degradation and conversion pathways, such as degradation of isoflavones (TOTTA *et al.* 2005) interference is possible. Individual differences in cytochrome enzyme activity or polymorphisms may cause variations in the metabolism of vitamin E and may lead to individual differences in the effectiveness of the catabolism of vitamin E and associated metabolite formation. The LCMs 9'- and 11'-COOH of γ-TOH and δ-TOH have been found only in sulfated form in plasma and liver in rats possibly because the sulfate group protects against degradation via β-oxidation, whereas the non-conjugated forms are rapidly conjugated (JIANG *et al.* 2007). In our study glucuronidase and sulfatase treatment has increased the amount of α-13'-COOH detectable in human serum (see Manuscript IV page 82). Therefore we conclude that this LCM occurs as a conjugate in human serum. In fact sulfotransferases and sulfatases are important for detoxification. Additionally they may be important for the accumulation of the LCM α-13'-COOH at least under supplementation conditions (JIANG *et al.* 2007). Next to the protective role of the sulfate group against β-oxidation regulatory functions have been described. For example for the diadzein sulfate metabolite which increases estrogen activity (TOTTA *et al.* 2005) and cholesterol the sulfate metabolite stabilizes cell membranes and interferes with signal transduction (JIANG *et al.* 2007; STROTT & HIGASHI 2003). Since vitamin E is an important component of cell membrane a sulfate metabolite may be also integrated into the cell membrane and may consequently stabilize the cell membrane. However, this hypothesis needs further investigations.

4.2 Intracellular transport of vitamin E metabolites during metabolic degradation

As described by Mustacich *et al.* the metabolism of vitamin E takes place in different cell compartments, starting at the endoplasmic reticulum, where the first LCM, α-13'-OH, is formed by CYP4F2/CYP3A4-dependent ω-hydroxylation. Further, the formation of the LCM α-13'-COOH and the following ICMs continues in peroxisomes. Subsequent degradation in mitochondria results in the formation of ICMs and SCMs (MUSTACICH *et al.* 2010). Since these catabolic steps occur in three different cellular compartments the formed metabolites have to be transported between these compartments. It is possible that different transporter and binding proteins exist which bind and transfer the metabolites specifically or unspecifically.

Since we detected the LCM of α-TOH, α-13-COOH, in human serum (WALLERT *et al.* 2013 // 2014) its transport from the liver to the circulation may be regulated somehow. Transport of α-TOH from the liver is carried out by α-TTP probably via VLDL which contain α-TOH and distribute it to non-hepatic tissues. However, the contribution of VLDL to the secretion of α-TOH is not completely understood since brefeldin A, an inhibitor disrupting the Golgi apparatus that also affect the secretion of VLDL, does not influence the secretion of α-TOH (ARITA *et al.* 1997). Our knowledge about the distribution of α-TOH may be incomplete, but nothing is known about the transport of its LCMs neither within the liver nor into the blood and extra-hepatic tissues. It is possible that the transport of α-13-COOH may occur via specific binding and transport proteins that have not yet been identified.

4.3 Long-chain metabolites of vitamin E as signaling molecules

Next to the possibility that vitamin E is metabolized to avoid its excessive accumulation it is possible that vitamin E procession may form metabolites which further act as molecular signals or protecting agents against cell damage caused by free radicals as known from α-TOH. Such effects have been described for the SCM of γ-TOH, γ-CEHC, which has antiproliferative activity (CARDENAS & GHOSH 2013; CONTE *et al.* 2004; GALLI *et al.* 2004b). This mayor SCM of vitamin E degradation reaches nanomolar concentrations in serum. The phenomenon that short-lived intermediate metabolites or end-products orchestrate signal transduction, regulate expression of metabolism-related genes and pathways is known, for example, for oxysterols, where the metabolites are also formed in nanomolar amounts (SCHROEPFER 2000), and prostaglandins which even act at picomolar levels (BELL-PARIKH *et al.* 2003). Additionally, isoprenes are known metabolites that have regulatory properties at very low cellular concentrations (FRITZ 2009).

Lipid mediators, such as ω3-derived lipoxins, resolvins and protectins, occur in 10-100 nM in human plasma (SCHROEPFER 2000) and have potent antiinflammatory, pro-resolving and tissue-protective effects (BUCKLEY *et al.* 2014). Resolvin E_1 is a metabolite of eicosapentaenoic

acid which regulates for example phagocytosis (HONG *et al.* 2008). Further, oxysterols, the oxidized derivates of cholesterol, function as signaling molecules that regulate a number of genes involved for example in cholesterol metabolism and homeostasis via LXR-α and LXR-β, SREBP1 and SREBP2 as well as HMG-CoA reductase activity (JANOWSKI *et al.* 1996; KANDUTSCH *et al.* 1978; LUND *et al.* 1998; RUSSELL 1999; SCHROEPFER 2000). This is in line with the so-called oxysterol hypothesis from Kandutsch *et al.* postulating that oxysterols and not cholesterol itself regulate cholesterol synthesis to a large extent (BJÖRKHEM & DICZFALUSY 2002; KANDUTSCH *et al.* 1978).

In contrast to the variety of investigations on vitamin E isomers, in particular α-TOH, our knowledge on LCMs derived from vitamin E and on their regulating activity is rare. Until now, only proapoptotic properties of the α-LCMs α-13'-OH and α-13'-COOH by activation of caspase-3 and 9 as well as poly (adenosine diphosphate (ADP)-ribose)polymerase (PARP)-1 cleavage through α-LCMs and δ-LCMs have been described in the human hepatocellular carcinoma cell line (HepG2) (BIRRINGER *et al.* 2010). Additionally, mitochondrial membrane potential is reduced whereas ROS formation is increased after treatment of HepG2 cells with these LCMs (BIRRINGER *et al.* 2010). Investigations in neutrophil and human promyelocytic leukemia cells showed that generation of LTB_4 is decreased by blocking of 5-LOX by δ-13'-COOH (JIANG *et al.* 2011). In addition to these oxidation- and ROS-related effects our research focuses the regulation of inflammation and maintenance of lipid homeostasis by the α-LCMs. As published recently both α-LCMs, α-13'-OH and α-13'-COOH, increased CD36 mRNA and protein expression, decreased phagocytosis in human macrophages in much lower concentrations and contrary to the metabolic precursor. But accumulation of lipids is regulated similarly by all compounds. In addition lipid metabolism related import, synthesis and export pathways were analyzed in several cell types and mostly regulations contrary to the effects of α-TOH have been observed. The PPARγ target adipophilin (ADRP) is a protein associated with triglyceride accumulation. Cytotoxicity caused by excessive accumulation of fatty acids can be diminished by PPARγ agonists (RABKIN *et al.* 2009). We found that the α-LCM α-13'-COOH increased the expression of ADRP in contrast to α-TOH (unpublished data), whereas stearic acid-related cytotoxicity decreased. Therefore we suggest that α-13'-COOH may protect macrophages from fatty acid-induced lipotoxicity and is possibly a PPARγ ligand. In addition key players of the LPS-induced inflammatory response of macrophages are blocked during α-13'-COOH treatment; this finding is in part consistent with results obtained with α-TOH (see Manuscript III page 51). Preliminary data indicate involving mitogen-activated protein kinases (MAPK) and other protein kinase (unpublished results).

It is worth to consider whether the physiological effects of intermediate catabolic products of vitamin E such as α-13'-COOH are limited because of their rapid metabolism which may be triggered by the metabolites themselves. This results in their rapid inactivation as it is known for oxysterols (KANDUTSCH *et al.* 1978). So far, except for the CEHCs, nothing is known about the kinetic profiles, stability of the metabolites or their regulatory potential. The catabolic end-product of α-TOH metabolism, α-CEHC, is mostly formed after excessive supplementation of *RRR*-α-TOH (TRABER 2013). But information on how far this metabolism is

and whether interindividual differences in time and dose of metabolite formation exist, are highly required for better understand the metabolism complexity and function of vitamin E. Until now, all proposed intermediate metabolites have been found in humans although not all have been detected so far. However, individual metabolite formation can vary due to differences in enzyme or receptor activity which in turn mediates formation and action of the metabolite. Oxysterols for example are known to autoregulate their metabolic fate (JANOWSKI *et al.* 1996). Since α-TOH supplementation leads to increased concentration of α-TOH, α-13-COOH and α-CEHC in plasma (SCHULTZ *et al.* 1995; WALLERT *et al.* 2013 // 2014), α-TOH metabolism may be induced by α-TOH or its metabolites. However, high-dose α-TOH injection in rats first increases plasma and liver α-TOH levels which are balanced by the massive excretion of the excessive α-TOH (MUSTACICH *et al.* 2006). Therefore two physiological mechanisms that allow the body to deal with excessive α-TOH are proposed: that the metabolites regulate their own formation via a feedback loop or trigger directly the excretion of α-TOH.

Depending on the context T3s are sometimes more potent than TOH in their regulatory activity. The degradation of T3 is quite similar to TOH as described in Chapter 1.1.3 except for the hydrogenisation of the double bonds in the side-chain and the kinetic of degradation. The half-lifes of TOH are about 10-20 times higher compared to that of the T3s, because of the faster degradation of T3s (KLUTH *et al.* 2005). Assuming that the metabolites formed are responsible for the biological activity of the vitamin E parents more rapid degradation of the T3s may also explain their higher biological activity. Since the LCMs and their metabolic precursors are structurally similar they may compete, for example, for membrane incorporation, transport and binding to receptor proteins and interference with signaling pathways. Possibly, the LCMs displace their precursors thus in causing more effective or even contrary regulations by the metabolites. This hypothesis may account for cellular and physiological effects regulated similarly by both TOH or T3 and their LCMs. For differences in their regulating behavior as it has been shown for CD36 by our group (WALLERT *et al.* 2013 // 2014), it is more likely that the LCMs have partly different or even contrary effects that are exclusively regulated by the metabolites in form of activating or inactivating precursor-independent signaling pathways.

4.4 Relationship of structure to effectiveness of α-TOH and their long-chain metabolites

Recent studies showed that α-TOH may modulate signal transduction and expression of genes involved in inflammation, proliferation and lipid metabolism to name a few. Although insufficient data exist to evaluate the biological activity of LCM of α-TOH (α-LCM), α-13'-OH and α-13'-COOH, first results suggest that differences in the regulation of scavenger receptors and inflammatory markers exist in comparison to α-TOH (WALLERT *et al.* 2013 // 2014), see Manuscript III page 51). Since α-TOH and its LCMs, α-13'-OH and α-13'-COOH, only differ in the terminal oxidative modification of the side-chain (see Figure 14), we

hypothesized that these hydroxyl and carboxyl groups are required for the recently described effects. Further, the eight main vitamin E isomers (α-, δ-, γ-, δ-TOH and T3) vary in their antioxidative capacity. Therefore we used the structurally related δ-LCMs δ-13'-OH and δ-13'-COOH, which lack two methyl groups at the hydroxychroman compared to α-13'-OH and α-13'-COOH, to perform whole-genome mRNA expression profiling in human macrophages. We found that the expression of about 650 genes was either up- or down-regulated significantly by more than 2-fold (unpublished data).

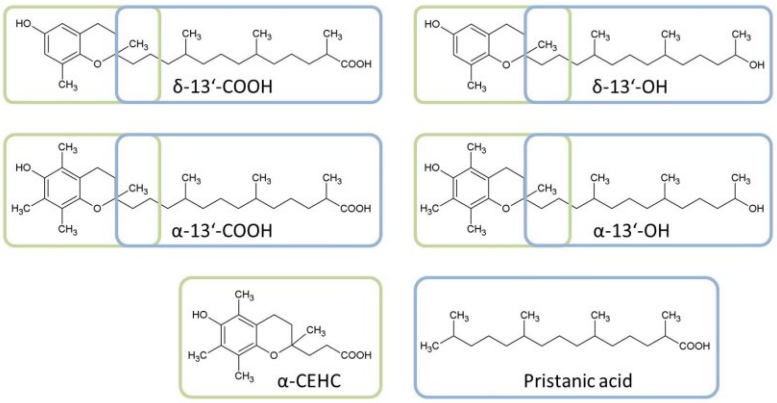


Figure 14: Molecular structures of interest.

The different α- and δ-LCMs caused almost identical patterns of changes in gene expression profiles. On the one hand we found genes regulated in the same way as the parent compounds α-TOH and δ-TOH and on the other hand contrary and further regulations were observed. With these whole-genome expression profiling studies we obtained for the first time a broad insight into biological pathways modulated by vitamin E and we confirmed that the metabolites exhibit considerable and distinct bioactivity *in vitro* (unpublished data). To support these findings, regulatory mechanisms of the metabolites and their structure-function relationship were investigated in more detail. We decided to investigate the δ-LCM instead of the γ-LCM although the γ-LCM would be of a particular and high interest because γ-TOH is the most important vitamin E isomer in US nutrition supplementation and the second most common TOH in human plasma that is known for its great biological potency (CAMPBELL *et al.* 2003). As for the α- and δ-LCMs the γ-LCMs are also not commercially available and the synthetic production is tricky and requires high amounts of garcinoic acid, which has been a limiting factor until now. A more accurate view at the metabolite structure reveals that the LCMs are comprised of the chromanol ring system and a branched-chain fatty acid-related side-chain. To evaluate the hypothesized structure-function comparison of the LCMs α-CEHC and pristanic acid were used as representatives of partial key structures (Figure 14). Hence pristanic acid, a branched-chain fatty acid, was used to characterize the contribution of the side-chains of α-13'-COOH and δ-13'-COOH. The contribution of the hydroxychroman ring system was investigated using the short-chain α-TOH metabolite α-CEHC. Furthermore, the δ-LCMs were used for a first insight into the contribution of the methylation patterns of the hydroxychroman ring system to the regulation of selected genes and protein expression (ADRP and CD36) in macrophages.

Adipophilin is a lipid droplet-associated protein involved in triglyceride storage in macrophages which protects the cells from lipotoxicity (BOSMA *et al.* 2012). We found that δ-13'-OH and δ-13'-COOH regulate ADRP, CD36 and triglyceride synthesis in macrophages to similar extent compared to the corresponding α-LCMs. Neither the partial structure of the

hydroxychroman ring nor the carboxylated side-chain, represented by α-CEHC and pristanic acid, respectively, regulate ADRP expression in concentrations similar to that of the LCMs. Both, the carboxyl and hydroxyl LCMs, were analyzed regarding the structure-function hypothesis. As the effectiveness of these LCMs was similar in our hands it was assumed that the terminal change of the side-chain *per se* and not the type of modification is critical for their activity. In contrast investigations by Birringer *et al.* showed significantly enhanced ROS formation and decreased mitochondrial membrane potential for α-13'-COOH but not for α-13'-OH (BIRRINGER *et al.* 2010). The oxidative status of the side-chain modification also determines the cytotoxic properties of the LCMs. The EC_{50} value in THP-1 macrophages is 7.4 µM for the carboxychromanol α-13'-COOH, whereas no EC_{50} value could be observed for the hydroxychromanol α-13'-OH (WALLERT *et al.* 2013 // 2014). Analysis of further compounds with multiple side-chain oxidations or non-oxidized side-chains such as nitration are therefore responsible. Since T3s are known to act more potently in some conditions compared to TOHs it could be important to investigate these structures in a comparable setting. Since the metabolites only differ in the T3-specific double bonds the importance of side-chain saturation in mediating biological activity could be ascertained. Further the investigation of both the side-chain saturation and the terminal oxidation status of the side-chain in parallel may be possible by using new amplexichromanol compounds (Figure 15) such as α-amplexichromanol, δ-E-amplexichromanol or δ-E-desoxyamplexichromanol (LAVAUD *et al.* 2013).

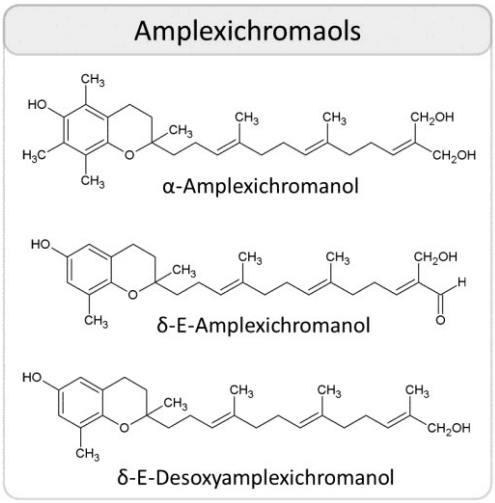


Figure 15: Amplexichromaols - Structures of interest for investigating structure-function-related effects.

Taken together these results provide clear evidence that both (i) the whole molecule comprised by the hydroxychroman ring and the side-chain, and (ii) the oxidation of the side-chain carrying a hydroxyl or carboxyl group is mandatory for the biological functions and specificity of these LCMs. These findings also suggest that a specific receptor exists which binds the α-LCMs and δ-LCMs, but neither the metabolic precursors α-TOH and δ-TOH nor the SCMs to modulate further signaling pathways. However, it is yet not clear, whether the ICMs still exhibit bioactivity similar to that of the LCMs.

4.5 Membrane incorporation of vitamin E and long-chain metabolites

Transversal diffusion is common for all lipophilic membrane components. Differences in transversal diffusion are known for vitamin E isomers but nothing has been described for the

metabolites so far. But cellular uptake by passive diffusion through the membrane is probably unlikely due to the chemical structure and size of the vitamin E molecules. Next to the transversal diffusion TOH and T3 are known to be incorporated into cell membranes and to be subject to lateral diffusion (Chapter 1.1.4.3). But nothing is known about diffusion processes of vitamin E metabolites yet.

Compared to 15 mol% cholesterol, for example in the Golgi apparatus, the amount of vitamin E in membranes is much lower, 0.1 mol% up to 1.5 mol% (SONNEN *et al.* 2005). Therefore the effects of TOHs and T3s on membrane fluidity may be entirely negligible at physiological concentrations (SONNEN *et al.* 2005). Additionally, vitamin E isomers diffuse lateral in biological membranes independently from their methylation pattern or the saturation of the isoprenoid side-chain, which suggests that the partly recommended higher antioxidant activity of T3s compared to TOHs is not based on the higher mobility of T3s (SONNEN *et al.* 2005). Furthermore, the cholesterol content decreases and the temperature increases lateral diffusion, respectively, whereas the influence of temperature is much more pronounced (SONNEN *et al.* 2005). The differences between TOHs and T3s in membranes are caused by the reorientational dynamics of T3s and a higher ordering effect of TOHs in the lipid phase (SONNEN *et al.* 2005). Membrane incorporation and lateral diffusion similar to vitamin E isomers may be possible for the LCM α-13'-OH and α-13'-COOH because of existing structural similarities to α-TOH. Regarding to the terminal oxidation possibly more space will be needed for α-13'-COOH in comparison to the non-oxidized precursor during the incorporation into the cell membrane. The side-chain of α-TOH has an unpolar terminal methyl group. In comparison the terminal carboxyl group of the α-13'-COOH is polar which probably requires more space within the membrane. It is conceivable that the space needed by α-13'-COOH within the membrane is between α-TOH and α-T3. Consequently the negative curvature stress caused by α-13'-COOH is probably also between both compounds (SEN *et al.* 2007).

The question is whether cellular uptake of the metabolites is required for affecting cellular signaling. Even in the case that the metabolites are not taken up by the cells this does not necessarily mean that the metabolites are unable to exert specific effects on cells. Binding of the metabolites to putative cell surface receptors may be sufficient to mediate outside-in signaling even if the compound is not taken up. Recently published data on the biological activity of the LCMs and further unpublished data outlined here provide evidence for a specific response of the cells to the metabolites and suggest that a receptor exists that specifically binds and mediates a specific regulatory response of the cells to the α-LCM (WALLERT *et al.* 2013 // 2014).

4.6 Does membrane-located receptor binding mediate the effects of the long-chain metabolites?

For several years vitamin E has been widely used as an antioxidant and protective agent to prevent cardiovascular diseases. Next to its antioxidative properties, vitamin E has been

suggested to modulate gene expression as summarized in Manuscript II (page 42). We suggest that the LCMs of vitamin E formed during vitamin E degradation represent a new class of signaling molecules mediating some of the effects seen with the parent compound on gene expression and cellular signaling (ZINGG 2007). But its function and mode of action is mostly unknown. *In vitro* studies on macrophages revealed that the metabolites affect expression of several genes as well as cellular processes such as scavenger receptor-mediated endocytosis and phagocytosis (WALLERT *et al.* 2013 // 2014). These results raise the question whether the metabolites may exhibit physiological functions not only in liver cells, where they were formed and detected first (BIRRINGER *et al.* 2010), but also in extra-hepatic tissues. Distribution of vitamin E, predominantly α-TOH, from the liver possibly by VLDL and its further transport to extra-hepatic tissues is widely known (for details see Chapter 1.1.2 of the introduction). But, transport of vitamin E metabolites has not yet been investigated. However, so far neither a specific receptor mediating the effects of vitamin E nor a physiological role of this vitamin, except for its function as a fertility factor in rats (EVANS & BISHOP 1922), have been unraveled. Therefore, the identification of a cellular receptor that specifically binds LCM and further mediates the regulatory signaling of the α-LCM is of particular interest.

Apart from α-TTP only TBP has been identified as cytosolic α-TOH binding protein, which facilitates incorporation of α-TOH into organelles and its transfer between membranes of intracellular organelles (DUTTA-ROY *et al.* 1993). Additionally human TAP is involved in lipid and TOH metabolism (ZINGG *et al.* 2010b) as well as intracellular TOH-related functions (STOCKER *et al.* 1999). Therefore it is obvious that a peripheral or integral receptor located at the cell surface or within the membrane may exists which either binds and further transfers the metabolites of vitamin E inside the cells to enable signaling function or mediates the signaling via pathways that need to be identified. This hypothesis is supported by findings obtained from primary human coronary artery smooth muscle cells (HCA-SMC) were the recombinant human tocopherol associated protein (hTAP) 1 may act as a sensor for lipids within the cell membrane. Subsequent transport of the lipids affords PI3K-mediated signaling and gene expression. α-Tocopherol and α-TP induce PI3Kγ-mediated signaling by binding of hTAP1, which further stimulates PI3Kγ activity (ZINGG *et al.* 2014). Vitamin E is acting probably by modulating specific and non-specific protein-membrane interactions, which enable further enzyme translocation to the plasma membrane, e.g. induced by phosphorylation, or by direct binding of the enzymes to receptors, structural proteins or transport proteins, such as lipoproteins or the hydrophobic ligand-binding proteins α-TTP and TBP (STOCKER *et al.* 1999; ZINGG 2007). As summarized by Zingg signal transduction mediated by vitamin E may be diverse and may lead to intracellular distribution of specific messenger lipids to enzymes and organelles (ZINGG 2007).

Vitamin E and its LCMs are lipid soluble compounds. Therefore they may traverse biological membranes by simple diffusion; in fact, however, they may require membrane receptors or transporters or a combination of both, as described for free fatty acids (BONEN *et al.* 1998; ZINGG 2007). Fatty acid transporters, such as plasma membrane located fatty acid binding protein (FABPpm), fatty acid translocase (FAT) and fatty acid transporter protein (FATP)

(BONEN *et al.* 1998) are potential transporters for vitamin E and its LCMs.

Since lipid G-protein-coupled receptors (GPCR) mediate several effects in cells such as cell migration, phagocyte activation by chemotaxis, degranulation, superoxide generation and vascular endothelial permeability (SUN & YE 2012). In these processes some genes are involved that are regulated by α-TOH (ZINGG *et al.* 2010b) and T3 (SYLVESTER *et al.* 2002). Therefore their involvement in LCM-mediated effects needs to be considered. Several GPCR agonists are known to trigger monocyte/macrophage inflammatory response, such as the platelet-activating factor (PAF) (LATTIN *et al.* 2007). In murine macrophages PAF antagonists inhibit LPS-induced iNos mRNA expression and nitric oxide production and reduce synthesis of nitric oxide in various animal models of endotoxemia (SZABÓ *et al.* 1993). As shown in Manuscript III α-13'-COOH blocks LPS-induced expression of iNos and Cox2 mRNA and protein as well as production of nitric oxide and PGE_2 in RAW264.7 macrophages. Furthermore LTB_4-activated nitric oxide production in *trypanosoma cruzi*-infected murine macrophages is mediated by the LTB_4 receptor-dependent pathway which is partially blocked by a PAF receptor antagonist UK-74505 (LATTIN *et al.* 2007; TALVANI *et al.* 2002). Based on these data it is possible that α-13'-COOH acts as a PAF antagonist and interferes with LTB_4, but this needs further investigation. Since GPCR signaling is responsible, for example, for alternative pathways of cell activation, such as TLR signaling activated by LPS (LATTIN *et al.* 2007), lipid soluble α-13'-COOH may act as a lipid mediator which binds to a GPCR (MURAKAMI 2011). This hypothesis is supported by findings obtained from peritoneal macrophages of heterotrimeric G protein knockout mice $G\alpha_{i2}^{-/-}$ show reduced production of inflammatory marker such as tumor necrosis factor (TNF)-α and thromboxane (TX) B_2 (FAN *et al.* 2005). In contrast stimulated splenocytes of these knockout mice showed increased production of inflammatory markers (FAN *et al.* 2005). Further, interaction of heterotrimeric G proteins and histone deacetylase (HDAC) type 5 has been shown (LATTIN *et al.* 2007; SPIEGELBERG & HAMM 2005). Histone deacetylases act as potent and selective negative regulators of the proinflammatory response in macrophages e.g. by blocking the ability of LPS to induce Cox2 mRNA in Hdac-8 overexpressing RAW264.7 macrophages (AUNG *et al.* 2006). In contrast, HDAC inhibitors induce hyperacetylation of histone H4 and stimulate expression of the proinflammatory cytokine IL-8 in SV-40 transformed bronchial epithelial cells (BEAS-2B) (TOMITA *et al.* 2003), whereas HDAC inhibitor-treated rats show reduced expression of IL-1β and TNFα (WANG *et al.* 2014). Taken together inconsistent data exist whether HDAC inhibitors enhance or decrease inflammatory response. However, vitamin E metabolites are structurally putative HDAC inhibitors (DASHWOOD & HO 2007). Since the α-LCMs block LPS-induced inflammatory response we investigated if the α-LCMs act as HDAC inhibitors *in vitro* (unpublished data). Since no data supporting this hypothesis have been obtained HDAC regulation mediated by α-LCM remain unclear.

G-protein coupled receptors are involved in several signaling pathways including PKA, PKB, PKC and phospholipase (PL) Cβ (Figure 16). Following the PLCβ pathway downstream, rat sarcoma (Ras), rapidly accelerated fibrosarcoma (Raf) and extracellular-signal regulated kinases (ERK1/2) are activated by phosphorylation. We already know that α-13'-COOH

enhances ERK1/2 phosphorylation in human fibroblasts. Starting from ERK1/2 subsequent cPLA phosphorylation is suggested. Since ERK1/2 is also regulated by PKA and subsequent Ras-related protein (Rap)/Raf signaling the involvement of PKA is assumed. Whereas PLCβ and PKA activate ERK1/2, PKC blocks it by interacting with Ras. Protein kinase B, also termed Akt, or further serine/threonine kinases maybe regulated through phosphatidylinositol-mediated signaling (BEAULIEU *et al.* 2009). α-Tocopherol or α-TP binding to CD36 may trigger

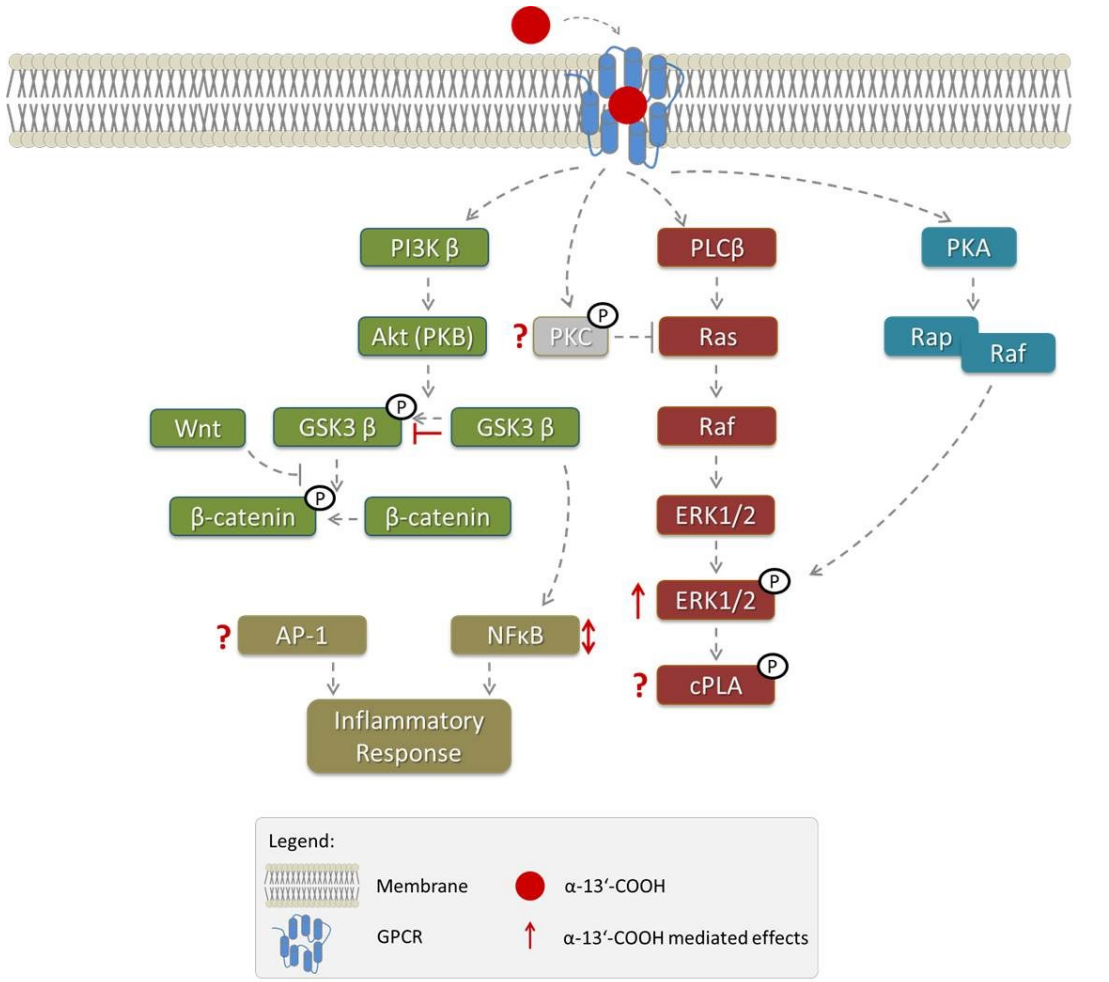


Figure 16: Suggested G-protein coupled receptor related signaling by α-13'-COOH.

phosphorylation of Akt (ZINGG *et al.* 2010a). Since α-13'-COOH upregulates CD36 (WALLERT *et al.* 2013 // 2014) the interaction of this LCM and Akt is suggested. First experiments gave a hint that glycogen synthase kinase (GSK) 3β phosphorylation at serine 9 is inhibited by α-13'-COOH (Figure 16, unpublished results). Glycogen synthase kinase 3β and the subunit GSK3α have been originally associated with the regulation of glycogen metabolism (FRAME & COHEN 2001). These kinases are constitutively active and can be inactivated by phosphorylation of single serine residues, serine 21 (GSK3α) and serine 9 (GSK3β), of their respective regulatory amino-terminal domains (BEAULIEU *et al.* 2009; FRAME & COHEN 2001). Since GSK3β is involved in phosphorylation and therefore destabilization of β-catenin it plays a key role in Wnt/β-catenin signaling via lymphoid enhancing factor (LEF) 1 and T cell factors (TCF) and subsequent signal transduction (WU &

PAN 2010). The possible involvement of α-13'-COOH in GSK3β signaling requires its interaction with the key signaling regulator of GSK3β Wnt. Active unphosphorylated GSK3β further activates NFκB, the main target of inflammatory response. Since we have shown that α-13'-COOH does not block LPS-induced NFκB p65 subunit translocation to the nucleus the blocking of GSK3β phosphorylation by α-13'-COOH has been hypothesized. In contrast, α-13'-COOH significantly reduced inflammatory markers such as Cox2 and iNos mRNA and protein expression as well as subsequent PGE_2 and nitric oxide production (see Manuscript III). One explanation can be the involvement of a further inflammation associated nuclear receptor named activator protein (AP)-1. The term AP-1 comprises homo- or heterodimeric protein complexes formed by Jun, Fos and cyclic AMP-dependent transcription factors (ATF). This is in good agreement with the fact that ERK1/2 and further MAPK targets regulate AP-1.

α-Tocopherol prevents macrophage foam cell formation by down-regulating scavenger receptor CD36 which is involved in the uptake of oxidized LDL (DEVARAJ *et al.* 2001; RICCIARELLI *et al.* 2000). Further, γ-TOH induces expression of adiponectin in C57BL/6J-mice at both transcriptional and circulating protein level (LANDRIER *et al.* 2009). Both genes are under control of PPARγ, a ligand-regulated transcription factor that plays an essential role in energy metabolism. *In vitro*, the amount of adiponectin mRNA was diminished in 3T3-L1 cells when coincubated with GW9662, a PPARγ antagonist. Two earlier studies demonstrated that γ-TOH activates PPARγ in the human colon cancer cell line SW480 (CAMPBELL *et al.* 2003), and induces expression of PPARγ and transglutaminase-1 protein (a target gene of PPARγ) in keratinocytes, respectively (De Pascale, M Clara *et al.* 2006). A paper by Fang *et al.* described T3 to activate different isoforms of PPARs thereby reducing blood glucose levels and insulin sensitivity of diabetic Db/Db mice (FANG *et al.* 2010). To sum up, existing studies indicate that all vitamin E isomers, except α-TOH, act as PPARγ agonists and induce PPARγ-dependent downstream mRNA and protein expression. Interestingly, α-TOH is the only vitamer which is not readily metabolized and excreted, and is the most abundant isomer in plasma. We therefore considered whether PPARγ activation is mediated by α-TOH metabolites, in particular LCMs rather than by α-TOH itself. As shown in Manuscript I the scavenger receptor CD36, the non-feedback regulated oxLDL importer, is up-regulated by both α-LCMs (page 33) and δ-LCMs (unpublished data). The initiation of signal transduction after binding to CD36 is known for oxLDL and fatty acids (ZINGG *et al.* 2010a). Upon binding of α-TP to CD36 direct CD36 ubiquitination or its internalization via endocytosis is possible (ZINGG *et al.* 2010a). Next to CD36 ADRP is regulated by the LCMs (unpublished data). Since these are target genes of the nuclear receptor PPARγ we suggested that the LCMs are natural PPARγ ligands. In contrast microarray analyses showed that other common PPARγ-related genes are not regulated. To evaluate the LCMs in detail regarding their PPAR isoform agonist activity profiling expression in *in vitro* studies or monitoring of their activity via luciferase-based promoter reporter assays are needed.

4.7 Problems and challenges of the *in vitro* studies

4.7.1 Challenge of reproduction of *in vitro* results

The reproducibility of results gained from both *in vitro* and *in vivo* studies is sometimes difficult. The reasons for this are diverse and may refer to the type (ZINGG *et al.* 2010a) and conditions of the cell culture, the used compounds (ZINGG *et al.* 2010b), the senescence of cells, spontaneous mutation or invisible contaminations, such as mycoplasmas within cell lines. Further, the supplier and the formulation of the compounds, duration time of experiments, and the performer may influence the results, to name only a few. Concentrations used to perform experiments are a highly discussed and important topic. In general physiological concentrations should be used, if possible. But a closer look to published data confirms that compliance to this general rule is rare. Therefore the physiological relevance of these data is dubious (ZINGG *et al.* 2010b). The use of higher concentrations for compounds *in vitro* than they occur physiologically *in vivo* is sometimes unavoidable. For example transport mechanisms occurring *in vivo* sometimes are not available in cell culture experiments, as discussed for α-TP uptake (ZINGG *et al.* 2010a), which justifies higher doses during incubation experiments in which to finally achieve comparable effective compound concentration within the cells.

If a new compound is investigated sometimes higher concentrations have to be used to get a first insight into the effects mediated by this compound. In the case of the newly identified LCM of α-TOH the best mode of application for the metabolite is not known. Therefore it needs to be investigated how different formulations may affect the 'bioactivity' and bioavailability of the metabolites in the cell culture as this may help to develop enhanced *in vitro* models that better reflect the situation *in vivo*. To closely imitate physiological conditions for lipid soluble compounds the application combined with serum or any other "carrier", such as lipids, lipoproteins or serum albumin is probably the best. During *in vitro* experiments an important point in general is the uptake of compounds into the cells. In the case of vitamin E isomers different protocols have been used, but not so far for its LCMs. The application of TOHs in cell culture experiments is frequently requested. Approved applications to ensure an adequate uptake of TOHs are coupling to serum (WU *et al.* 2005), or dissolving in either ethanol (RICCIARELLI *et al.* 2000) or dimethyl sulfoxide (DMSO) (BIRRINGER *et al.* 2010). Wu and colleagues have shown, for example, that TOH incubation in micromolar concentrations results in picomolar amounts of TOHs detectable in ECs (WU *et al.* 2005). We expect similar uptake mechanisms for TOH and LCMs for macrophages, but this needs further verification.

It is also not known which cellular levels of the metabolites can be achieved under the current experimental conditions (WALLERT *et al.* 2013 // 2014). However, significant differences in intra- and extracellular concentrations of metabolites are very well known. The LCMs of α-TOH were found only in relatively low concentrations in the cytoplasm of liver cells, but concentrations in organelles, such as microsomes, peroxisomes and mitochondria, are much higher (MUSTACICH *et al.* 2010). Since α-13'-COOH was measured in nanomolar

range in serum (WALLERT *et al.* 2013 // 2014), much higher concentrations up to lower micromolar range may occur at tissue or cellular level. Therefore it is probably appropriate to apply higher concentrations for cell culture experiments. Besides, it is a common observation that higher concentrations of bioactive molecules are required *in vitro* than they are likely available physiologically, as studies on regulatory features of oxysterols, prostaglandins and glitazones show (CHINETTI *et al.* 2001; JANOWSKI *et al.* 1996; TAM *et al.* 2006). In addition previously established conditions serve often as orientation for other research groups to achieve better comparability between results.

4.7.2 Species specific vitamin E metabolism – possible reasons for discrepancies of supplementation studies

Animal models have been used for decades as model systems to understand mechanisms and disease development in humans because of many similarities regarding metabolism and pathologies. Furthermore, breeding of knockout rodents for example yields highly useful disease models within an acceptable time which is not practicable in humans. For the evaluation of the developmental neurotoxicity in humans for example laboratory animals, rodents and non-human primates, are quite suitable in some cases, such as motivational/arousal, cognitive, motoric and social capacities (STANTON & SPEAR 1990). But, recent data suggest that animal models used to understand the initiation or progression of diseases in humans is likely not the best choice for all conditions (SEOK *et al.* 2013). Because of anatomical, physiological and developmental differences between species some effects observed in animals are not transferable to humans (ANDERSON *et al.* 2009). Therefore cross-species comparisons can cause problems including species differences in pharmacokinetics, developmental stage at exposure and assessment, or underlying mechanisms (STANTON & SPEAR 1990). Discrepancies can vary extremely dependent on the observed disease, end-point analysis, timing of sample acquisition and dosing of drugs (SEOK *et al.* 2013). Next to obvious differences, less striking species differences for example in peptide structure resulting in different substrate specificities between humans and mice have been described (KALUPOV *et al.* 2009). For several reasons previous studies have failed to systematically evaluate the suitability of murine clinical models mimicking human inflammatory diseases particularly in relation to the inflammatory response on the molecular level (SEOK *et al.* 2013). By analyzing published studies on the major pathways involved in the inflammatory response, Seok *et al.* focused high discrepancies between human and mice with respect to the individual gene activation in either direction or magnitude, such as the TLR pathways (SEOK *et al.* 2013).

Many studies elucidating vitamin E metabolism were performed in human cell lines (BIRRINGER *et al.* 2002b; SONTAG & PARKER 2002), mice (BARDOWELL *et al.* 2012; JOHNSON *et al.* 2012; KLUTH *et al.* 2005; MUSTACICH *et al.* 2009; ZHAO *et al.* 2010) or rats (MUSTACICH *et al.* 2006) and only a few in humans (ZHAO *et al.* 2010). Regarding the probably most important enzyme in vitamin E metabolism, the conserved ω-hydroxylase (PARKER & MCCORMICK 2005)

CYP4F2 in humans, Cyp4f14 was found in mice as an ortholog with >80% sequence identity (BARDOWELL *et al.* 2012). A second hydroxylase, CYP3A4 in humans or rather Cyp3a11 in mice (BARDOWELL *et al.* 2012), is suspected to be involved in TOH and T3 degradation.

Physiological basic α-TOH serum level in humans are around 25 µM and may reach after supplementation a maximum of 70 µM followed by a steady state of uptake and excretion (SCHULTZ *et al.* 1995). In contrast, Mustacich and co-workers found a maximum serum concentration of 483 µM after subcutaneous α-TOH injections for six days (10 mg/100 g body weight) in rats (MUSTACICH *et al.* 2006). Additionally the catabolic end-product α-CEHC increased up to 7.4 µM; this is several times higher as it is reachable in humans. Although subcutaneous injection has been used in many animal studies, it is not really comparable to oral supplementation and the differences in serum concentrations are enormous. After the sixth day of injection both α-TOH and α-CEHC serum and liver concentrations decreased steadily in the study of Mustacich *et al.* The authors hypothesized that enhanced metabolism and biliary excretion, as it is known for humans, are reasons for this counterbalancing that correlates with the accumulation of metabolic intermediates. In parallel the first hepatic LCM formed in the catabolism of α-TOH, α-13'-OH, with is detectable in rats at baseline, increased up to the third day and subsequently decreased after reaching a steady state within the next days. The SCM 5'-α-CMBHC behaves similarly following supplementation, with the difference that it was not detected at baseline (MUSTACICH *et al.* 2006).

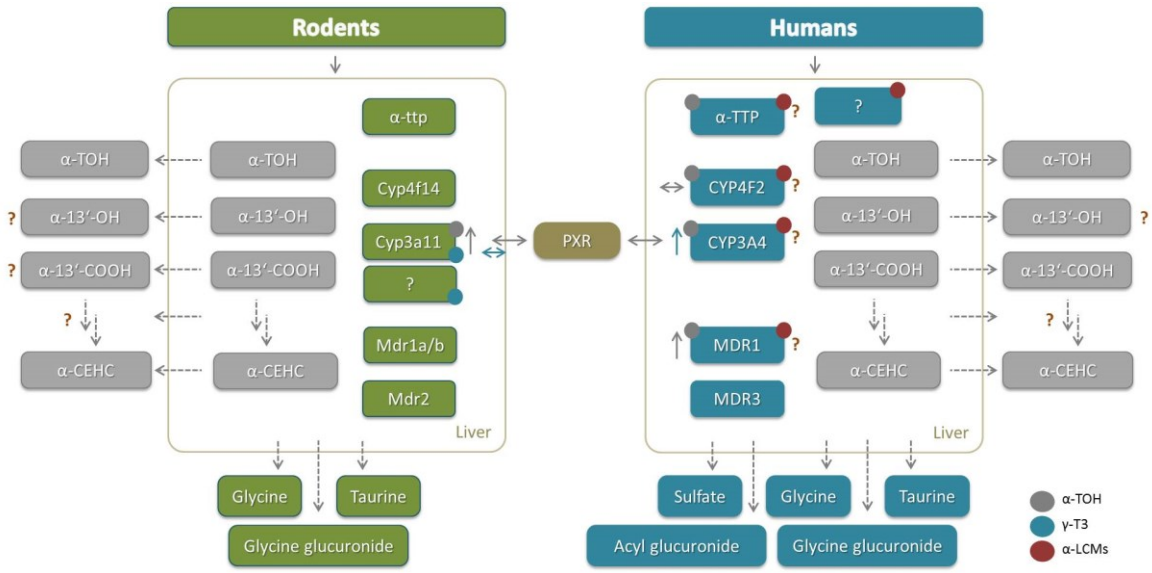


Figure 17: Similarities and differences in human and rodent vitamin E metabolism. In general the metabolism of vitamin E in rodents and humans is processed by similar enzymes: α-TTP is the main binding protein for vitamin E in the respective species, CYPs for the formation of LCMs, and MRD family proteins are responsible for excretion of conjugated catabolic end-products mainly via urine. Metabolites formed by ω-hydroxylation and oxidation were detected in the liver and partly in serum of rodents and humans. The regulation of the vitamin E metabolism by PXR binding is suggested. (JOHNSON *et al.* 2012; KLUTH *et al.* 2005; MUSTACICH *et al.* 2009; WALLERT *et al.* 2013 // 2014)

The exporter MDR1 and the murine ortholog Cyp3a11 of the human CYP3A4 hepatic enzyme (MUSTACICH *et al.* 2006; MUSTACICH *et al.* 2009) increased under supplementation with α-TOH in mice (MUSTACICH *et al.* 2009) and rats (MUSTACICH *et al.* 2006), but α-TTP and CYP4F2 were not regulated (Figure 17). Whereas humans and rats have two MDR isomers, MDR1 and MDR3, mice have Mdr1a and Mdr1b and the MDR3 ortholog Mdr2 which is important for basal biliary α-TOH excretion (MUSTACICH *et al.* 1998; MUSTACICH *et al.* 2009). Mustacich and coworkers conclude that this regulation shows that apart from some species-related discrepancies the α-TOH mechanism seems to be conserved across species (MUSTACICH *et al.* 2009). But some data exist suggesting that this cannot be generalized for all vitamin E isomers. γ-Tocotrienol, for example, induced CYP3A4 in human HepG2 cells but not the analogous Cyp3a11 mRNA in mice liver possibly because activation of the PXRs, which is involved in vitamin E metabolism, shows species-specific differences (KLUTH *et al.* 2005) (see Figure 17). It has been suggested further that in mice Cyp3a11 might not be the enzyme which metabolizes γ-T3 (KLUTH *et al.* 2005). Therefore the participation of CYP4F2 needs to be investigated in more detail (KLUTH *et al.* 2005).

Vitamin E catabolism results in the urinary metabolite CEHC which is mostly conjugated. Recently metabolites α-CEHC glycine, α-CEHC glycine glucuronide, and α-CEHC taurine were found in both mice and humans, whereas α-CEHC sulfate and α-CEHC acyl glucuronide were present in human urine only (JOHNSON *et al.* 2012). This further shows the differences between murine and human vitamin E metabolism (JOHNSON *et al.* 2012) (Figure 17). However, there is a large interindividual variability in α-TOH metabolism in humans, too. Whereas α-CEHC glucuronide and sulfate are metabolites that are always detected, α-CEHC taurine, α-CEHC glycine, and α-CEHC acyl glucuronide were present in about 50-90% of the investigated volunteers (JOHNSON *et al.* 2012). Johnson and coworkers concluded that polymorphisms in drug-metabolizing enzymes and transporters and/or competitive co-metabolism of endogenous metabolites by gut microflora may be the reason for these differences (JOHNSON *et al.* 2012). To what extent the formation of the LCMs is subject to individual variations needs further investigations. Individual pharmacokinetics and differences in formation of these metabolites in addition to the latest findings by our group (BIRRINGER *et al.* 2010; JIANG *et al.* 2008; WALLERT *et al.* 2013 // 2014) suggest a physiological and probably pathophysiological importance for the metabolites. To answer some open questions, supplementation studies with naturally-occurring *RRR*-α-TOH over weeks using different doses to study saturation kinetics of the metabolite in human plasma and the half-life time and plasma levels of the LCMs have to be performed. First data for α-13'-COOH suggest that plasma levels of this LCM vary following supplementation in individuals (unpublished data), which may be very important compared to the fact that α-TOH levels are known for its small variations. This is of further fundamental importance when discussing the influences of vitamin E on pathological conditions or rather the progression of disease such as atherogenesis and diabetes and how vitamin E acts on the systemic and cellular level. Hence, the ratio of the metabolite and α-TOH plasma concentrations may be relevant. Studies in humans after one week supplementation with 1000 IU/d α-TOH showed a fivefold increase in LCM α-13'-COOH concentration in serum (unpublished data), whereas α-TOH

increased only up to 3-fold suggesting higher relevance for the metabolite after supplementation. Further findings about maximum plasma levels of α-13'-COOH or if a steady state is achieved after supplementation may give a hint for its physiological relevance. Both healthy humans and AVED patients, characterized by an α-TTP deficiency resulting in lower α-TOH serum level due to disturbed α-TOH distribution, are of particular interest for these metabolic studies. Since nothing is known about the release of α-13'-COOH into the serum analyzing blood of AVED patients may give a hint whether α-TTP acts as a transport protein for this metabolite. In this case the serum levels of α-13'-COOH will not increase independently from α-TOH, if nonetheless the level increase after vitamin E supplementation further transport proteins or mechanisms distinct from α-TTP have to be proposed for the transport of LCM and their release into the blood.

Independent of the investigated species conclusions or recommendations should be based on more than one disease model, to provide adequate information and representations of disease progression (MURRAY & FRENK 2008). In this context Fiume and coworkers reported that α-TOH absorption studies and retention in serum did not show satisfactory correlation between human and rats which caused underestimation of the potency of free TOH in humans (FIUME 2002). Development of alternative artificial human models to replace animal studies for *in vitro* studies to investigate disease-related cell types or tissues has been promoted over the last years (HUH *et al.* 2010).

5 Summary

Atherosclerosis and its complications such as stroke and myocardial infarction are next to cancer the leading cause of death in Western industrialized societies. In Germany about 40% of total mortality in 2012 was related to cardiovascular events (Statistisches Bundesamt 2012). This will increase further in the next years (HONARBAKHSH & SCHACHTER 2009). Use of vitamin E for prevention of cardiovascular diseases (CVD) was first mentioned in the 1950's by Dr. Even Shut (CHAN 1998). Since that time, vitamin E has been known as an antioxidant and has been investigated for decades in regard to these properties. Many studies showed that vitamin E exhibits antioxidative capacity *in vitro* (VAN DAM *et al.* 2003). Since age-related diseases, such as CVD, cancer and Alzheimer disease, are associated with oxidative events, vitamin E intake was promising in preventing these diseases or at least slowing down their progression. Based on promising results in *in vitro* and animal studies several large-scale human intervention studies were initiated and followed up over years. In regard to preventing cardiovascular complications these studies revealed controversial results and failed to demonstrate clear invers correlations or positive effects of vitamin E supplementation (WALLERT *et al.* 2014). Reasons for the poor outcomes are complex. Apart from differences in study designs, such as the selection of volunteers, the sizes of cohorts, doses and duration of supplementation and application form of vitamin E, individual differences in vitamin E metabolism may be responsible for different outcomes. The importance of the metabolic differences needs to be clarified further.

The term vitamin E describes a group of isomers within α-, β-, γ-, δ-tocopherol (TOH) and the respective tocotrienols are the most important ones. The isomers differ in methylation patterns of the hydroxychromal ring system and saturation of the side-chain. In the liver vitamin E metabolism occurs in three compartments: endoplasmic reticulum (ER), peroxisomes and mitochondria. In ER the long-chain metabolite (LCM) α-13'-OH is formed by CYP4F2/CYP3A4-dependent ω-hydroxylation followed by α-oxidation which results in the formation of α-13'-COOH. Two subsequent β-oxidation steps in peroxisomes and three β-oxidation steps in mitochondria form ICMs and finally SCMs which are mainly excreted via urine. Although many of these metabolites have been detected in humans, nearly nothing is known about their physiological or pathophysiological relevance.

We detected considerable amounts of the LCM α-13'-COOH in serum of healthy humans by LC/MS-QTOF. Due to optimization of the preparation protocols regarding enzymatic processing and organic extraction we detected α-13'-COOH in human serum in nanomolar concentrations. This finding provides evidence for physiological relevance of this LCM. After one week of supplementation with 1000 IU/d *RRR*-α-TOH the concentration of α-13'-COOH increased at least fivefold. We assume that the formation and the effects of α-13'-COOH are different between individuals, which could possibly explain the controversial outcomes from human intervention studies.

Apart from antioxidative properties further independent effects of α-TOH have been

reported (AZZI *et al.* 1995). Therefore we investigated the effects of the α-LCMs, which have been synthesized from the African bitter nut *garcinia kola*, for antioxidative independent effects. As macrophages play a key role in inflammation and atherosclerosis, human and murine macrophages were used as model systems to investigate the molecular modes of action of α-TOH and the respective LCMs α-13'-COOH and α-13'-OH.

Scavenger receptor CD36 takes up modified lipoproteins and plays therefore a key role in maintaining cellular lipid homeostasis (DEVARAJ *et al.* 2001). By using quantitative real-time PCR and flow cytometry we found an induction of CD36 mRNA and protein expression by α-13'-OH and α-13'-COOH, whereas the precursor α-TOH decreased both kinds of expression. Pretreatment of macrophages with the LCMs followed by oxidized LDL (oxLDL) incubation significantly increased the oxLDL-related CD36 protein expression, whereas α-TOH did not. Apart from the scavenger receptor-mediated uptake, oxLDL particles can be internalized by macrophages via phagocytosis. Flow cytometric analyses with fluorescent microbeads showed that the LCMs inhibited phagocytosis in contrast to α-TOH (IZGÜT-UYSAL *et al.* 2004). Although the LCMs increased CD36 expression net uptake of fluorescent oxLDL and accumulation of neutral lipids were decreased by α-13'-OH, α-13'-COOH and α-TOH, possibly by a balancing of the lipid import mechanisms involving CD36 receptor and phagocytosis (WALLERT *et al.* 2013 // 2014).

Next to foam cell formation, inflammation plays a pivotal role in atherogenesis. Investigations of bacterial lipopolysaccharide (LPS)-activated murine macrophages by using quantitative real time PCR showed that LPS-triggered upregulation of key inflammatory markers, such as cyclooxygenase 2 (Cox2) and inducible nitric oxide synthase (iNos), were significantly blocked by α-13'-COOH, but not by α-TOH treatment. Similar results were obtained from Western blotting analysis for Cox2 and iNos protein. Since Cox2 regulates the arachidonic acid cascade the downstream formation of prostaglandin E_2 (PGE_2) was analyzed by ELISA. As expected α-13'-COOH, but also α-TOH, reduced LPS-induced production of PGE_2. In parallel iNos regulates the production of nitric oxide from L-arginine. Since expression of iNos was blocked by α-13'-COOH, the production of nitric oxide was also inhibited. In contrast no significant changes after α-TOH treatment occurred. The inflammatory response is mainly mediated by activation of transcription factor NFκB. However, neither α-13'-COOH nor α-TOH blocked the LPS-induced translocation of the p65 subunit of NFκB into the nucleus. Therefore we suggest that modulation of the inflammatory response by α-13'-COOH may be independent from translocation of the NFκB subunit p65.

In summary LCMs of α-TOH modulate lipid metabolism and exhibit antiinflammatory properties more effectively and by involving different signaling pathways compared to the metabolic precursor α-TOH. We therefore suggest that the LCMs represent a new class of regulatory signaling molecules. With these results we achieved a decisive contribution to clarify the biological mode of action of vitamin E, which is not well understood yet despite many years of research. Therefore further investigations are necessary to unravel the mode of action of the newly identified physiological α-TOH-metabolites.

6 Zusammenfassung

Die Atherosklerose mit ihren Komplikationen Herzinfarkt und Schlaganfall zählt in westlichen Industrienationen zu den häufigsten Todesursachen. Im Jahre 2012 bedingten kardiovaskuläre Erkrankungen (CVD) ca. 40% aller Todesfälle in Deutschland (Statistisches Bundesamt 2012); die Tendenz ist steigend (HONARBAKHSH & SCHACHTER 2009). Vitamin E in der Prävention von CVD wurde erstmalig in den 1950er Jahren durch den Mediziner Dr. Even Shut beschrieben (CHAN 1998). Schon damals wurden die antioxidativen Eigenschaften des Vitamin E erkannt, die daraufhin in den folgenden Jahrzehnten im Fokus der Vitamin E-Forschung standen. Zahlreiche *in vitro* und im Tiermodell durchgeführte Studien ließen einen positiven Effekt des Vitamin E hinsichtlich altersbedingter Erkrankungen, wie z.B. CVD, Krebs und Alzheimer, vermuten. Viele Wissenschaftler erhofften sich daher, diese Erkrankungen durch die nutritive Zufuhr oder die Supplementation mit Vitamin E in ihrer Entstehung zu unterdrücken oder zumindest deren Krankheitsverlauf mildern zu können. In den letzten Jahrzehnten wurden daher zahlreiche Humanstudien durchgeführt, die jedoch mit sehr unterschiedlichen Ergebnissen endeten. Trotz der *in vitro* gezeigten atheroprotektiven Eigenschaften (VAN DAM *et al.* 2003) hatte eine Supplementierung mit Vitamin E keinen eindeutig positiven Effekt auf die Prävalenz koronarer Herzerkrankungen (WALLERT *et al.* 2014). Die teils kontroversen Ergebnisse der Humanstudien begründen sich nicht nur in sehr unterschiedlichen Studiendesigns (bspw. Größe und Art der Probandengruppen, Dosis, Dauer sowie Art der Vitamin E-Supplementation). Möglicherweise ist der Metabolismus von Vitamin E im menschlichen Organismus individuell sehr unterschiedlich. Die Bedeutung dieser Unterschiede für die Entstehung von Erkrankungen ist jedoch bis heute noch nicht vollständig verstanden.

Vitamin E ist eine hauptsächlich aus α-, β-, γ-, δ-Isomeren der Tocopherole (TOH) und Tocotrienole bestehende Mischung. Die Isomere unterscheiden sich durch ihr Methylierungsmuster am Chromanolringsystem sowie der Sättigung der Seitenkette. Vitamin E wird im humanen Organismus in der Leber abgebaut. Dieser Katabolismus erfolgt in drei Kompartimenten: am endoplasmatischen Retikulum (ER), den Peroxisomen und den Mitochondrien. Am ER wird zunächst durch eine CYP4F2/CYP3A4-abhängige ω-Hydroxylierung der langkettige Metabolit (LCM) α-13‘-OH gebildet, gefolgt von einer α-Oxidation zu α-13‘-COOH. Weiterführend erfolgen zwei β-Oxidationsschritte in den Peroxisomen und drei in den Mitochondrien. Dies führt zum Abbau der Seitenkette und somit zur Bildung der mittelkettigen und schließlich der kurzkettigen wasserlöslichen Metabolite, die vorrangig mit dem Urin ausgeschieden werden. Bis heute wurden zahlreiche dieser Metabolite im humanen Organismus detektiert; physiologische und mögliche pathophysiologische Effekte dieser Metabolite sind jedoch weitgehend unbekannt.

Es ist uns erstmalig gelungen, den LCM α-13‘-COOH im humanen Serum von gesunden Probanden mittels LC/MS-QTOF zu identifizieren. Durch eine Optimierung der Probenaufarbeitung hinsichtlich enzymatischer Verdauung und der verwendeten Extraktionsmittel konnten wir diesen LCM in nanomolaren Konzentrationen im Serum

detektieren. Nach einer einwöchigen Supplementation mit 1000 IU/d *RRR*-α-TOH konnte ein Anstieg von α-13‘-COOH um den Faktor fünf beobachtet werden. Wir vermuten weiterhin, dass die Bildung und Wirkung dieser LCMs individuell sehr unterschiedlich ist; dies könnte eine Erklärung für die kontroversen Ergebnisse der Humanstudien sein. Diese Daten geben erste Hinweise auf eine mögliche physiologische Relevanz des Metaboliten α-13‘-COOH.

Neben der Wirkung als Antioxidans besitzt Vitamin E auch nicht-antioxidative Eigenschaften (AZZI *et al.* 1995). Wir untersuchten daher die LCMs des α-TOHs, welche aus der afrikanischen Bitternuss *garcinia kola* synthetisiert werden, im Hinblick auf diese unabhängigen Eigenschaften. Makrophagen sind von besonderer Bedeutung für inflammatorische Prozesse und die Atherosklerose. Daher wurden in der vorliegenden Arbeit humane und murine Makrophagen verwendet, um neue Wirkmechanismen von Vitamin E und seinen Metaboliten zu untersuchen.

Für das Aufrechterhalten der Lipidhomöostase spielt der *scavenger*-Rezeptor CD36 aufgrund seiner Beteiligung an der Aufnahme modifizierter Lipoproteine eine entscheidende Rolle (DEVARAJ *et al.* 2001). Mittels quantitativer Echtzeit-RT-PCR (RT-qPCR) und Durchflusszytometrie konnte gezeigt werden, dass die Expression von CD36-mRNA und -Protein durch α-13‘-OH und α-13‘-COOH signifikant induziert wird, wohingegen die Ausgangssubstanz α-TOH eine Senkung bewirkt. Eine Vorinkubation mit den LCMs gefolgt von einer zeitgleichen Applikation der Metabolite mit oxLDL zeigte eine signifikante additive Steigerung der CD36-Proteinexpression, wohingegen die Inkubation mit α-TOH keine Veränderung der oxLDL-induzierten CD36-Proteinexpression ergab. Ein weiterer Mechanismus für die Aufnahme von modifizierten LDL ist die Phagozytose. In Phagozytose-Assays mit fluoreszenzmarkierten Mikropartikeln konnte mittels Durchflusszytometrie gezeigt werden, dass auch hier eine konträre Regulation zu α-TOHs erfolgt (IZGÜT-UYSAL *et al.* 2004), die LCM inhibieren die Phagozytose. Trotz der induzierten CD36-Expression senken die LCMs jedoch im Vergleich zu α-TOH die Aufnahme von fluoreszenzmarkierten oxLDL sowie die Akkumulation von Neutrallipiden. Möglicherweise erfolgt eine Kompensation der CD36-vermittelten Aufnahme von oxLDL durch die verminderte Phagozytose (WALLERT *et al.* 2013 // 2014).

Neben der Schaumzellbindung sind Entzündungsreaktionen entscheidend für die Initiation und Progression der Atherosklerose. Durch Untersuchungen der Genexpression mittels RT-qPCR konnten wir zeigen, dass die Stimulation der Inflammationsmarker Cyclooxygenase 2 (Cox2) und induzierbare Stickstoffmonoxid-Synthase (iNos) durch bakterielle Lipopolysaccharide (LPS) durch α-13‘-COOH signifikant gehemmt werden, jedoch nicht durch α-TOH. Dieser Befund spiegelt sich auch auf Proteinebene wieder. Da die Cox2 die Arachidonsäure-Kaskade reguliert, ist eine Regulation der Cox2-Proteinmenge mit der Produktion von Prostaglandin E_2 (PGE_2) assoziiert. ELISA bestätigten, dass α-13‘-COOH, aber auch α-TOH, die LPS-induzierte Produktion von PGE_2 signifikant hemmen. Parallel dazu reguliert die iNos die Produktion von Stickstoffmonoxid aus L-Arginin. Wie zu erwarten führt die Hemmung der iNos-Proteinmenge zu einer signifikanten Hemmung der Produktion von Stickstoffmonoxid durch α-13‘-COOH, jedoch nicht durch α-TOH. Ein zentraler Regulator

inflammatorischer Prozesse ist der Transkriptionsfaktor NFκB. Daher wurde der Einfluss auf die Translokation der p65-Untereinheit von NFκB in den Zellkern untersucht. Weder durch α-13‘-COOH noch durch α-TOH konnte die LPS-induzierte Translokation der p65-Untereinheit geblockt werden. Daher ist zu vermuten, dass α-13‘-COOH die durch LPS-stimulierte Entzündungsreaktion unabhängig von der Translokation der NFκB p65-Untereinheit hemmt.

Zusammenfassend kann festgestellt werden, dass die langkettigen Metabolite, bezogen auf den Lipidstoffwechsel und ihre antiinflammatorische Wirkung, biologisch deutlich aktiver als der metabolische Ausgangsstoff α-TOH sind. Weiterhin erfolgt die Vermittlung dieser athero-protektiven Effekte vermutlich über andere Signalwege als im Fall von α-TOH. Ferner deuten weitere Ergebnisse dieser Doktorarbeit darauf hin, dass die α-LCM eine neue Klasse von regulatorisch wirkenden Signalmolekülen darstellen könnten. Mit diesen Ergebnissen könnte ein entscheidender neuer Beitrag zur weiterführenden Aufklärung der biologischen Funktion von Vitamin E erzielt werden. Trotz jahrzehntelanger Forschung ist diese nach wie vor nicht eindeutig geklärt. Weiterführende Experimente sind nun notwendig, um die den Beobachtungen zugrundliegenden Wirkmechanismen der α-LCM aufzuklären.

A References

1. Abe C, Uchida T, Ohta M, Ichikawa T, Yamashita K, Ikeda S. Cytochrome P450-dependent metabolism of vitamin E isoforms is a critical determinant of their tissue concentrations in rats. *Lipids*, **2007**; 42(7); 637-645.
2. Afri M, Ehrenberg B, Talmon Y, Schmidt J, Cohen Y, Frimer AA. Active oxygen chemistry within the liposomal bilayer. Part III: Locating Vitamin E, ubiquinol and ubiquinone and their derivatives in the lipid bilayer. *Chem Phys Lipids,* **2004***; 131(1)*; 107-121.
3. Aguglia U, Annesi G, Pasquinelli G, Spadafora P, Gambardella A, Annesi F, Pasqua AA, Cavalcanti F, Crescibene L, Bagalà A, Bono F, Oliveri RL, Valentino P, Zappia M, Quattrone A. Vitamin E deficiency due to chylomicron retention disease in Marinesco-Sjögren syndrome. *Ann Neurol,* **2000***; 47(2)*; 260-264.
4. Anderson T, Khan NK, Tassinari MS, Hurtt ME. Comparative juvenile safety testing of new therapeutic candidates: relevance of laboratory animal data to children. *J Toxicol Sci,* **2009***; 34 Suppl 2*; SP209-15.
5. Arita M, Nomura K, Arai H, Inoue K. alpha-tocopherol transfer protein stimulates the secretion of alpha-tocopherol from a cultured liver cell line through a brefeldin A-insensitive pathway. *Proc Natl Acad Sci U.S.A.,* **1997***; 94(23)*; 12437-12441.
6. Arlt S, Müller-Thomsen T, Beisiegel U, Kontush A. Effect of one-year vitamin C- and E-supplementation on cerebrospinal fluid oxidation parameters and clinical course in Alzheimer's disease. *Neurochem Res,* **2012***; 37(12)*; 2706-2714.
7. Ascherio A, Rimm EB, Hernán MA, Giovannucci E, Kawachi I, Stampfer MJ, Willett WC. Relation of consumption of vitamin E, vitamin C, and carotenoids to risk for stroke among men in the United States. *Ann Intern Med,* **1999***; 130(12)*; 963-970.
8. Asplund K. Antioxidant vitamins in the prevention of cardiovascular disease: a systematic review. *J Intern Med,* **2002***; 251(5)*; 372-392.
9. Atkinson J, Harroun T, Wassall SR, Stillwell W, Katsaras J. The location and behavior of alpha-tocopherol in membranes. *Mol Nutr Food Res,* **2010***; 54(5)*; 641-651.
10. Aung HT, Schroder K, Himes SR, Brion K, van Zuylen W, Trieu A, Suzuki H, Hayashizaki Y, Hume DA, Sweet MJ, Ravasi T. LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. *FASEB J.,* **2006***; 20(9)*; 1315-1327.
11. Axford-Gately RA, Wilson GJ. Myocardial infarct size reduction by single high dose or repeated low dose vitamin E supplementation in rabbits. *Can J Cardiol,* **1993***; 9(1)*; 94-98.
12. Azzi A. The role of alpha-tocopherol in preventing disease. *Eur J Nutr,* **2004***; 43 Suppl 1(0)*; I/18-25.
13. Azzi A. Molecular mechanism of alpha-tocopherol action. *Free Radic Biol Med,* **2007***; 43(1)*; 16-21.
14. Azzi A, Boscoboinik D, Clément S, Marilley D, Ozer NK, Ricciarelli R, Tasinato A. Alpha-tocopherol as a modulator of smooth muscle cell proliferation. *Prostaglandins Leukot Essent Fatty Acids,* **1997***; 57(4-5)*; 507-514.

15. Azzi A, Boscoboinik D, Marilley D, Ozer NK, Stäuble B, Tasinato A. Vitamin E: a sensor and an information transducer of the cell oxidation state. *Am J Clin Nutr,* **1995***; 62(6 Suppl)*; 1337S-1346S.

16. Azzi A, Gysin R, Kempná P, Munteanu A, Negis Y, Villacorta L, Visarius T, Zingg J. Vitamin E mediates cell signaling and regulation of gene expression. *Ann N Y Acad Sci,* **2004***; 1031*; 86-95.

17. Azzi A, Ricciarelli R, Zingg JM. Non-antioxidant molecular functions of alpha-tocopherol (vitamin E). *FEBS Lett.,* **2002***; 519(1-3)*; 8-10.

18. Bäck M, Ketelhuth DFJ, Agewall S. Matrix Metalloproteinases in Atherothrombosis. *Prog Cardiovas Dis,* **2010***; 52(5)*; 410-428.

19. Baker H, Frank O, DeAngelis B, Feingold S. Plasma tocopherol in man at various times after ingesting free or acetylated tocopherol. *Nutr Rep Int,* **1980***(21)*; 531-536.

20. Bardowell SA, Duan F, Manor D, Swanson JE, Parker RS. Disruption of Mouse Cytochrome P450 4f14 (Cyp4f14 Gene) Causes Severe Perturbations in Vitamin E Metabolism. *J Biol Chem,* **2012***; 287(31)*; 26077-26086.

21. Barella L, Muller PY, Schlachter M, Hunziker W, Stöcklin E, Spitzer V, Meier N, Pascual-Teresa S de, Minihane A, Rimbach G. Identification of hepatic molecular mechanisms of action of alpha-tocopherol using global gene expression profile analysis in rats. *Biochim Biophys Acta,* **2004***; 1689(1)*; 66-74.

22. Beaulieu J, Gainetdinov RR, Caron MG. Akt/GSK3 signaling in the action of psychotropic drugs. *Annu Rev Pharmacol Toxicol,* **2009***; 49*; 327-347.

23. Behrens WA, Madère R. Alpha- and gamma tocopherol concentrations in human serum. *J Am Coll Nutr,* **1986***; 5(1)*; 91-96.

24. Behrens WA, Thompson JN, Madère R. Distribution of alpha-tocopherol in human plasma lipoproteins. *Am J Clin Nutr,* **1982***; 35(4)*; 691–696.

25. Bell-Parikh LC, Ide T, Lawson JA, McNamara P, Reilly M, FitzGerald GA. Biosynthesis of 15-deoxy-delta12,14-PGJ2 and the ligation of PPARgamma. *J Clin Invest,* **2003***; 112(6)*; 945-955.

26. Berdnikovs S, Abdala-Valencia H, McCary C, Somand M, Cole R, Garcia A, Bryce P, Cook-Mills JM. Isoforms of vitamin E have opposing immunoregulatory functions during inflammation by regulating leukocyte recruitment. *J Immunol,* **2009***; 182(7)*; 4395-4405.

27. Betancor-Fernandez A, Sies H, Stahl W, Polidori MC. In vitro antioxidant activity of 2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman (alpha-CEHC), a vitamin E metabolite. *Free Radic Res,* **2002***; 36(8)*; 915-921.

28. Bieri JG, Wu AL, Tolliver TJ. Reduced intestinal absorption of vitamin E by low dietary levels of retinoic acid in rats. *J Nutr,* **1981***; 111(3)*; 458-467.

29. Biesalski HK, Köhrle J, Schürmann K (Hrsg.). Vitamine, Spurenelemente und Mineralstoffe. Thieme, Stuttgart, **2002**.

30. Birringer M. Analysis of vitamin E metabolites in biological specimen. *Mol Nutr Food Res,* **2010***; 54(5)*; 588-598.

31. Birringer M, Drogan D, Brigelius-Flohé R. Tocopherols are metabolized in HepG2 cells by side chain omega-oxidation and consecutive beta-oxidation. *Free Radic Biol Med,* **2001***; 31(2)*; 226-232.

32. Birringer M, EyTina JH, Salvatore BA, Neuzil J. Vitamin E analogues as inducers of apoptosis: structure-function relation. *Br. J. Cancer,* **2003***; 88(12)*; 1948-1955.

33. Birringer M, Lington D, Vertuani S, Manfredini S, Scharlau D, Glei M, Ristow M. Proapoptotic effects of long-chain vitamin E metabolites in HepG2 cells are mediated by oxidative stress. *Free Radic Biol Med,* **2010***; 49(8)*; 1315-1322.

34. Birringer M, Pfluger P, Kluth D, Landes N, Brigelius-Flohé R. Identities and differences in the metabolism of tocotrienols and tocopherols in HepG2 cells. *J Nutr,* **2002a***; 132(10)*; 3113-3118.

35. Birringer M, Pfluger P, Kluth D, Landes N, Brigelius-Flohé R. Identities and differences in the metabolism of tocotrienols and tocopherols in HepG2 cells. *J Nutr,* **2002b***; 132(10)*; 3113-3118.

36. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol,* **2010***; 11(10)*; 889-896.

37. Björkhem I, Diczfalusy U. Oxysterols: friends, foes, or just fellow passengers? *Arterioscler Thromb Vasc Biol,* **2002***; 22(5)*; 734-742.

38. Bjørneboe A, Bjørneboe GE, Bodd E, Hagen BF, Kveseth N, Drevon CA. Transport and distribution of alpha-tocopherol in lymph, serum and liver cells in rats. *Biochim Biophys Acta,* **1986***; 889(3)*; 310-315.

39. Bjørneboe A, Bjørneboe GE, Drevon CA. Absorption, transport and distribution of vitamin E. *J Nutr,* **1990***; 120(3)*; 233-242.

40. Blot WJ, Li JY, Taylor PR, Guo W, Dawsey SM, Li B. The Linxian trials: mortality rates by vitamin-mineral intervention group. *Am J Clin Nutr,* **1995***; 62(6 Suppl)*; 1424S-1426S.

41. Boaz M, Smetana S, Weinstein T, Matas Z, Gafter U, Iaina A, Knecht A, Weissgarten Y, Brunner D, Fainaru M, Green MS. Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. *Lancet,* **2000***; 356(9237)*; 1213-1218.

42. Bobák M, Brunner E, Miller NJ, Skodová Z, Marmot M. Could antioxidants play a role in high rates of coronary heart disease in the Czech Republic? *Eur J Clin Nutr,* **1998***; 52(9)*; 632-636.

43. Bonen A, Dyck DJ, Luiken JJ. Skeletal muscle fatty acid transport and transporters. *Adv Exp Med Biol,* **1998***; 441*; 193-205.

44. Borel P, Grolier P, Mekki N, Boirie Y, Rochette Y, Le Roy B, Alexandre-Gouabau MC, Lairon D, Azais-Braesco V. Low and high responders to pharmacological doses of beta-carotene: proportion in the population, mechanisms involved and consequences on beta-carotene metabolism. *J Lipid Res,* **1998***; 39(11)*; 2250-2260.

45. Borel P, Pasquier B, Armand M, Tyssandier V, Grolier P, Alexandre-Gouabau MC, Andre M, Senft M, Peyrot J, Jaussan V, Lairon D, Azais-Braesco V. Processing of vitamin A and E in the human gastrointestinal tract. *Am. J. Physiol. Gastrointest. Liver Physiol,* **2001***; 280(1)*; G95-G103.

46. Boscoboinik D, Szewczyk A, Azzi A. Alpha-tocopherol (vitamin E) regulates vascular smooth muscle cell proliferation and protein kinase C activity. *Arch Biochem Biophys,* **1991***; 286(1)*; 264-269.

47. Bosma M, Hesselink, Matthijs KC, Sparks LM, Timmers S, Ferraz MJoão, Mattijssen F, van Beurden D, Schaart G, de Baets, Marc H, Verheyen FK, Kersten S, Schrauwen P. Perilipin 2 improves insulin sensitivity in skeletal muscle despite elevated intramuscular lipid levels. *Diabetes,* **2012***; 61(11)*; 2679-2690.

48. Bouayed J, Bohn T. Exogenous antioxidants - Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxid Med Cell Longev,* **2010***; 3(4)*; 228-237.

49. Brewer GJ. Why vitamin E therapy fails for treatment of Alzheimer's disease. *J Alzheimers Dis,* **2010***; 19(1)*; 27-30.

50. Brigelius-Flohé R. Vitamin E: the shrew waiting to be tamed. **2009**.

51. Brigelius-Flohé R, Kelly FJ, Salonen JT, Neuzil J, Zingg J, Azzi A. The European perspective on vitamin E: current knowledge and future research. *Am J Clin Nutr,* **2002***; 76(4)*; 703-716.

52. Brigelius-Flohé R, Traber MG. Vitamin E: function and metabolism. *FASEB J,* **1999***; 13(10)*; 1145-1155.

53. Brown BG, Zhao XQ, Chait A, Fisher LD, Cheung MC, Morse JS, Dowdy AA, Marino EK, Bolson EL, Alaupovic P, Frohlich J, Albers JJ. Simvastatin and niacin, antioxidant vitamins, or the combination for the prevention of coronary disease. *N Engl J Med,* **2001***; 345(22)*; 1583-1592.

54. Brown ED, Micozzi MS, Craft NE, Bieri JG, Beecher G, Edwards BK, Rose A, Taylor PR, Smith JC. Plasma carotenoids in normal men after a single ingestion of vegetables or purified beta-carotene. *Am J Clin Nutr,* **1989***; 49(6)*; 1258-1265.

55. Brown MS, Ho YK, Goldstein JL. The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters. *J Biol Chem,* **1980***; 255(19)*; 9344-9352.

56. Buckley CD, Gilroy DW, Serhan CN. Proresolving Lipid Mediators and Mechanisms in the Resolution of Acute Inflammation. *Immunity,* **2014***; 40(3)*; 315-327.

57. Buhman KF, Accad M, Farese RV. Mammalian acyl-CoA:cholesterol acyltransferases. *Biochim Biophys Acta,* **2000***; 1529(1-3)*; 142-154.

58. Burton GW, Ingold KU. Vitamin E as an in vitro and in vivo antioxidant. *Ann N Y Acad Sci,* **1989***; 570*; 7-22.

59. Burton GW, Ingold KU, Foster DO, Cheng SC, Webb A, Hughes L, Lusztyk E. Comparison of free alpha-tocopherol and alpha-tocopheryl acetate as sources of vitamin E in rats and humans. *Lipids,* **1988***; 23(9)*; 834-840.

60. Buss H, Dörrie A, Schmitz ML, Frank R, Livingstone M, Resch K, Kracht M. Phosphorylation of serine 468 by GSK-3beta negatively regulates basal p65 NF-kappaB activity. *J Biol Chem,* **2004***; 279(48)*; 49571-49574.

61. Campbell SE, Stone WL, Whaley SG, Qui M, Krishnan K. Gamma (gamma) tocopherol upregulates peroxisome proliferator activated receptor (PPAR) gamma (gamma) expression in SW 480 human colon cancer cell lines. *BMC Cancer,* **2003***; 3*; 25.

62. Cardenas E, Ghosh R. Vitamin E: A dark horse at the crossroad of cancer management. *Biochem Pharmacol,* **2013***; 86(7)*; 845-852.

63. Catalán U, Fernández-Castillejo S, Pons L, Heras M, Aragonés G, Anglès N, Morelló J, Solà R. Alpha-tocopherol and BAY 11-7082 reduce vascular cell adhesion molecule in human aortic endothelial cells. *J Vasc Res,* **2012***; 49(4)*; 319-328.

64. Chan AC. Vitamin E and atherosclerosis. *J Nutr,* **1998***; 128(10)*; 1593-1596.

65. Cheon JH, Kim JS, Kim JM, Kim N, Jung HChae, Song IS. Plant sterol guggulsterone inhibits nuclear factor-kappaB signaling in intestinal epithelial cells by blocking IkappaB kinase and ameliorates acute murine colitis. *Inflamm Bowel Dis,* **2006***; 12(12)*; 1152-1161.

66. Chiku S, Hamamura K, Nakamura T. Novel urinary metabolite of d-delta-tocopherol in rats. *J Lipid Res,* **1984***; 25(1)*; 40-48.

67. Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, Teissier E, Minnich A, Jaye M, Duverger N, Brewer HB, Fruchart JC, Clavey V, Staels B. PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat Med,* **2001***; 7(1)*; 53-58.

68. Chinetti-Gbaguidi G, Baron M, Bouhlel M, Vanhoutte J, Copin C, Sebti Y, Derudas B, Mayi T, Bories G, Tailleux A, Haulon S, Zawadzki C, Jude B, Staels B. Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARγ and LXRα pathways. *Circ Res,* **2011***; 108(8)*; 985-995.

69. Chinetti-Gbaguidi G, Staels B. Macrophage polarization in metabolic disorders. *Curr Opin Lipidol,* **2011***; 22(5)*; 365-372.

70. Cho J, Kang DW, Ma X, Ahn S, Krausz KW, Luecke H, Idle JR, Gonzalez FJ. Metabolomics reveals a novel vitamin E metabolite and attenuated vitamin E metabolism upon PXR activation. *J Lipid Res,* **2009***; 50(5)*; 924-937.

71. Chun J, Lee J, Ye L, Exler J, Eitenmiller Ronald R. Tocopherol and tocotrienol contents of raw and processed fruits and vegetables in the United States diet. *J Food Comp Anal,* **2006***(19)*; 196-204.

72. Clément SA, Tan CC, Guo J, Kitta K, Suzuki YJ. Roles of protein kinase C and alpha-tocopherol in regulation of signal transduction for GATA-4 phosphorylation in HL-1 cardiac muscle cells. *Free Radic Biol Med,* **2002***; 32(4)*; 341-349.

73. Collot-Teixeira S, Martin J, McDermott-Roe C, Poston R, McGregor JL. CD36 and macrophages in atherosclerosis. *Cardiovasc Res,* **2007***; 75(3)*; 468-477.

74. Conte C, Floridi A, Aisa C, Piroddi M, Floridi A, Galli F. Gamma-tocotrienol metabolism and antiproliferative effect in prostate cancer cells. *Ann N Y Acad Sci,* **2004***; 1031*; 391-394.

75. Cook NR, Albert CM, Gaziano JM, Zaharris E, MacFadyen J, Danielson E, Buring JE, Manson JE. A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: results from the Women's Antioxidant Cardiovascular Study. *Arch Intern Med,* **2007***; 167(15)*; 1610-1618.

76. Cook-Mills JM, McCary CA. Isoforms of vitamin E differentially regulate inflammation. *Endocr Metab Immune Disord Drug Targets,* **2010***; 10(4)*; 348-366.

77. Cordero Z, Drogan D, Weikert C, Boeing H. Vitamin E and risk of cardiovascular diseases: a review of epidemiologic and clinical trial studies. *Crit Rev Food Sci Nutr,* **2010***; 50(5)*; 420-440.

78. Cullen P, Rauterberg J, Lorkowski S. The pathogenesis of atherosclerosis. *Handb Exp Pharmacol,* **2005***(170)*; 3-70.

79. Cuschieri J, Bulger E, Biligren J, Garcia I, Maier RV. Vitamin E inhibits endotoxin-mediated transport of phosphatases to lipid rafts. *Shock,* **2007***; 27(1)*; 19-24.

80. Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci U.S.A.,* **1990***; 87(13)*; 5134-5138.

81. Dashwood RH, Ho E. Dietary histone deacetylase inhibitors: from cells to mice to man. *Semin Cancer Biol,* **2007***; 17(5)*; 363-369.

82. David AR, Kershaw A, Heagerty A. Atherosclerosis and diet in ancient Egypt. *Lancet,* **2010***; 375(9716)*; 718-719.

83. De Pascale MC, Bassi AM, Patrone V, Villacorta L, Azzi A, Zingg J. Increased expression of transglutaminase-1 and PPARgamma after vitamin E treatment in human keratinocytes. *Arch Biochem Biophys,* **2006***; 447(2)*; 97-106.

84. Delport R, Ubbink JB, Human JA, Becker PJ, Myburgh DP, Vermaak WJ. Antioxidant vitamins and coronary artery disease risk in South African males. *Clin Chim Acta,* **1998***; 278(1)*; 55-60.

85. Devaraj S, Hugou I, Jialal I. Alpha-tocopherol decreases CD36 expression in human monocyte-derived macrophages. *J Lipid Res,* **2001***; 42(4)*; 521-527.

86. Diplock AT, Lucy JA. The biochemical modes of action of vitamin e and selenium: A hypothesis. **1973**.

87. Doi K, Matsuura M, Kawara A, Tanaka T, Baba S. Influence of dietary fiber (konjac mannan) on absorption of vitamin B12 and vitamin E. *Tohoku J Exp Med,* **1983***; 141 Suppl*; 677-681.

88. Dong L, Low P, Dyason JC, Wang X, Prochazka L, Witting PK, Freeman R, Swettenham E, Valis K, Liu J, Zobalova R, Turanek J, Spitz DR, Domann FE, Scheffler IE, Ralph SJ, Neuzil J. α-Tocopheryl succinate induces apoptosis by targeting ubiquinone-binding sites in mitochondrial respiratory complex II. *Oncogene,* **2008***; 27(31)*; 4324-4335.

89. Dutta A, Dutta SK. Vitamin E and its role in the prevention of atherosclerosis and carcinogenesis: a review. *J Am Coll Nutr,* **2003***; 22(4)*; 258-268.

90. Dutta-Roy AK, Leishman DJ, Gordon MJ, Campbell FM, Duthie GG. Identification of a low molecular mass (14.2 kDa) alpha-tocopherol-binding protein in the cytosol of rat liver and heart. *Biochem Biophys Res Commun,* **1993***; 196(3)*; 1108-1112.

91. Dwyer JT, Woteki C, Bailey R, Britten P, Carriquiry A, Gaine PC, Miller D, Moshfegh A, Murphy MM, Smith Edge M. Fortification: new findings and implications. *Nutr Rev,* **2014***; 72(2)*; 127-141.

92. Eidelman RS, Hollar D, Hebert PR, Lamas GA, Hennekens CH. Randomized trials of vitamin E in the treatment and prevention of cardiovascular disease. *Arch Intern Med,* **2004***; 164(14)*; 1552-1556.

93. Ekstrand-Hammarström B, Osterlund C, Lilliehöök B, Bucht A. Vitamin E down-modulates mitogen-activated protein kinases, nuclear factor-kappaB and inflammatory responses in lung epithelial cells. *Clin Exp Immunol,* **2007***; 147(2)*; 359-369.

94. El-Sohemy A, Baylin A, Spiegelman D, Ascherio A, Campos H. Dietary and adipose tissue gamma-tocopherol and risk of myocardial infarction. *Epidemiology,* **2002***; 13(2)*; 216-223.

95. Erwig L, Henson PM. Clearance of apoptotic cells by phagocytes. *Cell Death Differ,* **2008***; 15(2)*; 243-250.

96. Evans HM, Bishop KS. On the existence of a hitherto unrecognized dietary factor essential for reproduction. *Science,* **1922***; 56(1458)*; 650-651.

97. Evans RW, Shaten BJ, Day BW, Kuller LH. Prospective association between lipid soluble antioxidants and coronary heart disease in men. The Multiple Risk Factor Intervention Trial. *Am J Epidemiol,* **1998***; 147(2)*; 180-186.

98. Fan H, Zingarelli B, Peck OM, Teti G, Tempel GE, Halushka PV, Spicher K, Boulay G, Birnbaumer L, Cook JA. Lipopolysaccharide- and gram-positive bacteria-induced cellular inflammatory responses: role of heterotrimeric Galpha(i) proteins. *Am J Physiol, Cell Physiol,* **2005***; 289(2)*; C293-301.

99. Fang F, Kang Z, Wong C. Vitamin E tocotrienols improve insulin sensitivity through activating peroxisome proliferator-activated receptors. *Mol Nutr Food Res,* **2010***; 54(3)*; 345-352.

100. Farina N, Isaac, Mokhtar Gad El Kareem Nasr, Clark AR, Rusted J, Tabet N. Vitamin E for Alzheimer's dementia and mild cognitive impairment. *Cochrane Database Syst Rev,* **2012***; 11*; CD002854.

101. Farley SM, Leonard SW, Taylor AW, Birringer M, Edson KZ, Rettie AE, Traber MG. ω-Hydroxylation of phylloquinone by CYP4F2 is not increased by α-tocopherol. *Mol Nutr Food Res,* **2013**.

102. Faruqi R, de la Motte C, DiCorleto PE. Alpha-tocopherol inhibits agonist-induced monocytic cell adhesion to cultured human endothelial cells. *J Clin Invest,* **1994***; 94(2)*; 592-600.

103. Feig JE, Parathath S, Rong JX, Mick SL, Vengrenyuk Y, Grauer L, Young SG, Fisher EA. Reversal of hyperlipidemia with a genetic switch favorably affects the content and inflammatory state of macrophages in atherosclerotic plaques. *Circulation,* **2011***; 123(9)*; 989-998.

104. Feki M, Souissi M, Mokhtar E, Hsairi M, Kaabachi N, Antebi H, Alcindor LG, Mechmeche R, Mebazaa A. Vitamin E and coronary heart disease in Tunisians. *Clin Chem,* **2000***; 46(9)*; 1401-1405.

105. Ferns G, Williams J, Forster L, Tull S, Starkey B, Gershlick A. Cholesterol standardized plasma vitamin E levels are reduced in patients with severe angina pectoris. *Int J Exp Pathol,* **2000***; 81(1)*; 57-62.

106. Fitzgerald ML, Mujawar Z, Tamehiro N. ABC transporters, atherosclerosis and inflammation. *Atherosclerosis,* **2010***; 211(2)*; 361-370.

107. Fiume MZ. Final Report on the Safety Assessment of Tocopherol, Tocopheryl Acetate, Tocopheryl Linoleate, Tocopheryl Linoleate/Oleate, Tocopheryl Nicotinate, Tocopheryl

Succinate, Dioleyl Tocopheryl Methylsilanol, Potassium Ascorbyl Tocopheryl Phosphate, and Tocophersolan. *Int J Toxicol,* **2002***; 21(6)*; 51-116.

108. Fortmann SP, Burda BU, Senger CA, Lin JS, Whitlock EP. Vitamin and mineral supplements in the primary prevention of cardiovascular disease and cancer: An updated systematic evidence review for the U.S. Preventive Services Task Force. *Ann Intern Med,* **2013***; 159(12)*; 824-834.

109. Frame S, Cohen P. GSK3 takes centre stage more than 20 years after its discovery. *Biochem J,* **2001***; 359(Pt 1)*; 1-16.

110. Frei B, Gaziano JM. Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation. *J Lipid Res,* **1993***; 34(12)*; 2135-2145.

111. Freiser H, Jiang Q. Gamma-tocotrienol and gamma-tocopherol are primarily metabolized to conjugated 2-(beta-carboxyethyl)-6-hydroxy-2,7,8-trimethylchroman and sulfated long-chain carboxychromanols in rats. *J Nutr,* **2009a***; 139(5)*; 884-889.

112. Freiser H, Jiang Q. Optimization of the enzymatic hydrolysis and analysis of plasma conjugated gamma-CEHC and sulfated long-chain carboxychromanols, metabolites of vitamin E. *Anal Biochem,* **2009b***; 388(2)*; 260-265.

113. Fritz G. Targeting the mevalonate pathway for improved anticancer therapy. *Curr Cancer Drug Targets,* **2009***; 9(5)*; 626-638.

114. Fujisawa A, Dunlap WC, Yamamoto Y. Vitamin E protection in the biochemical adaptation of marine organisms to cold-water environments. *Comp Biochem Physiol B: Biochem Mol Biol,* **2010***; 157(2)*; 145-158.

115. Gaetano G de. Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. Collaborative Group of the Primary Prevention Project. *Lancet,* **2001***; 357(9250)*; 89-95.

116. Galli F, Lee R, Dunster C, Kelly FJ. Gas chromatography mass spectrometry analysis of carboxyethyl-hydroxychroman metabolites of alpha- and gamma-tocopherol in human plasma. *Free Radic Biol Med,* **2002***; 32(4)*; 333-340.

117. Galli F, Piroddi M, Lannone A, Pagliarani S, Tomasi A, Floridi A. A comparison between the antioxidant and peroxynitrite-scavenging functions of the vitamin E metabolites alpha- and gamma-carboxyethyl-6-hydroxychromans. *Int J Vitam Nutr Res,* **2004a***; 74(5)*; 362-373.

118. Galli F, Stabile AM, Betti M, Conte C, Pistilli A, Rende M, Floridi A, Azzi A. The effect of α- and γ-tocopherol and their carboxyethyl hydroxychroman metabolites on prostate cancer cell proliferation. *Arch Biochem Biophys,* **2004b***; 423(1)*; 97-102.

119. Ghosh S, Zhao B, Bie J, Song J. Macrophage cholesteryl ester mobilization and atherosclerosis. *Vascul Pharmacol,* **2010***; 52(1-2)*; 1-10.

120. Gianello R, Libinaki R, Azzi A, Gavin PD, Negis Y, Zingg J, Holt P, Keah H, Griffey A, Smallridge A, West SM, Ogru E. Alpha-tocopheryl phosphate: a novel, natural form of vitamin E. *Free Radic Biol Med,* **2005***; 39(7)*; 970-976.

121. Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U.S.A.,* **1979a***; 76(1)*; 333-337.

122. Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U.S.A.,* **1979b***; 76(1)*; 333-337.

123. González R, Collado JA, Nell S, Briceño J, Tamayo MJ, Fraga E, Bernardos A, López-Cillero P, Pascussi JM, Rufián S, Vilarem M, De la Mata M, Brigelius-Flohe R, Maurel P, Muntané J. Cytoprotective properties of alpha-tocopherol are related to gene regulation in cultured D-galactosamine-treated human hepatocytes. *Free Radic Biol Med,* **2007***; 43(10)*; 1439-1452.

124. Gordon N. Hereditary vitamin-E deficiency. *Dev Med Child Neurol,* **2001***; 43(2)*; 133-135.

125. Gotoh N, Watanabe H, Oka T, Mashimo D, Noguchi N, Hata K, Wada S. Dietary Marine-Derived Tocopherol has a Higher Biological Availability in Mice Relative to Alpha-Tocopherol. *Lipids,* **2009***; 44(2)*; 133-143.

126. Grammas P, Hamdheydari L, Benaksas EJ, Mou S, Pye QN, Wechter WJ, Floyd RA, Stewart C, Hensley K. Anti-inflammatory effects of tocopherol metabolites. *Biochem Bioph Res Co,* **2004***; 319(3)*; 1047-1052.

127. Grebenstein N, Schumacher M, Graeve L, Frank J. α-Tocopherol transfer protein is not required for the discrimination against γ-tocopherol in vivo but protects it from side-chain degradation in vitro. *Mol Nutr Food Res,* **2014**; n/a.

128. Gruppo Italiano per lo Studio della. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Sopravvivenza nell'Infarto miocardico. *Lancet,* **1999***; 354(9177)*; 447-455.

129. Guo L, Song Z, Li M, Wu Q, Wang D, Feng H, Bernard P, Daugherty A, Huang B, Li X. Scavenger Receptor BI Protects against Septic Death through Its Role in Modulating Inflammatory Response. *J Biol Chem,* **2009***; 284(30)*; 19826-19834.

130. Hacquebard M, Carpentier YA. Vitamin E: absorption, plasma transport and cell uptake. *Curr Opin Clin Nutr Metab Care,* **2005***; 8(2)*; 133-138.

131. Halstead BW, Zwickl CM, Morgan RE, Monteith DK, Thomas CE, Bowers RK, Berridge BR. A clinical flow cytometric biomarker strategy: validation of peripheral leukocyte phospholipidosis using Nile red. *J Appl Toxicol,* **2006***; 26(2)*; 169-177.

132. Hansson GK, Hermansson A. The immune system in atherosclerosis. *Nat Immunol,* **2011***; 12(3)*; 204-212.

133. Hardwick JP. Cytochrome P450 omega hydroxylase (CYP4) function in fatty acid metabolism and metabolic diseases. *Biochem Pharmacol,* **2008***; 75(12)*; 2263-2275.

134. Harel M, Aharoni A, Gaidukov L, Brumshtein B, Khersonsky O, Meged R, Dvir H, Ravelli, Raimond B G, McCarthy A, Toker L, Silman I, Sussman JL, Tawfik DS. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nat Struct Mol Biol,* **2004***; 11(5)*; 412-419.

135. Hasty AH, Gruen ML, Terry ES, Surmi BK, Atkinson RD, Gao L, Morrow JD. Effects of vitamin E on oxidative stress and atherosclerosis in an obese hyperlipidemic mouse model. *J Nutr Biochem,* **2007***; 18(2)*; 127-133.

136. Hathcock JN, Azzi A, Blumberg J, Bray T, Dickinson A, Frei B, Jialal I, Johnston CS, Kelly FJ, Kraemer K, Packer L, Parthasarathy S, Sies H, Traber MG. Vitamins E and C are safe across a broad range of intakes. *Am J Clin Nutr,* **2005***; 81(4)*; 736-745.

137. Havel RJ, EDER HA, BRAGDON JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest,* **1955***; 34(9)*; 1345-1353.

138. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20 536 high-risk individuals: a randomised placebo-controlled trial // MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet,* **2002***; 360(9326)*; 23-33.

139. Hense HW, Stender M, Bors W, Keil U. Lack of an association between serum vitamin E and myocardial infarction in a population with high vitamin E levels. *Atherosclerosis,* **1993***; 103(1)*; 21-28.

140. Hensley K, Benaksas EJ, Bolli R, Comp P, Grammas P, Hamdheydari L, Mou S, Pye QN, Stoddard MF, Wallis G, Williamson KS, West M, Wechter WJ, Floyd RA. New perspectives on vitamin E: gamma-tocopherol and carboxyelthylhydroxychroman metabolites in biology and medicine. *Free Radic Biol Med,* **2004***; 36(1)*; 1-15.

141. Hercberg S, Galan P, Preziosi P, Bertrais S, Mennen L, Malvy D, Roussel A, Favier A, Briançon S. The SU.VI.MAX Study: a randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. *Arch Intern Med,* **2004***; 164(21)*; 2335-2342.

142. Herz J, Qiu SQ, Oesterle A, DeSilva HV, Shafi S, Havel RJ. Initial hepatic removal of chylomicron remnants is unaffected but endocytosis is delayed in mice lacking the low density lipoprotein receptor. *Proc Natl Acad Sci U.S.A.,* **1995***; 92(10)*; 4611-4615.

143. Hirvonen T, Virtamo J, Korhonen P, Albanes D, Pietinen P. Intake of flavonoids, carotenoids, vitamins C and E, and risk of stroke in male smokers. *Stroke,* **2000***; 31(10)*; 2301-2306.

144. Hoeksema MA, Stöger JL, de Winther, Menno PJ. Molecular pathways regulating macrophage polarization: implications for atherosclerosis. *Curr Atheroscler Rep,* **2012***; 14(3)*; 254-263.

145. Honarbakhsh S, Schachter M. Vitamins and cardiovascular disease. *Br J Nutr,* **2009***; 101(8)*; 1113-1131.

146. Hong S, Porter TF, Lu Y, Oh SF, Pillai PS, Serhan CN. Resolvin E1 metabolome in local inactivation during inflammation-resolution. *J Immunol,* **2008***; 180(5)*; 3512-3519.

147. Horwitt MK, Harvey CC, Dahm CH, Searcy MT. Relationship between tocopherol and serum lipid levels for determination of nutritional adequacy. *Ann N Y Acad Sci,* **1972***; 203*; 223-236.

148. Hosomi A, Arita M, Sato Y, Kiyose C, Ueda T, Igarashi O, Arai H, Inoue K. Affinity for alpha-tocopherol transfer protein as a determinant of the biological activities of vitamin E analogs. *FEBS Lett,* **1997***; 409(1)*; 105-108.

149. Hsu M, Savas U, Griffin KJ, Johnson EF. Human cytochrome p450 family 4 enzymes: function, genetic variation and regulation. *Drug Metab Rev,* **2007***; 39(2-3)*; 515-538.

150. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. Reconstituting organ-level lung functions on a chip. *Science,* **2010***; 328(5986)*; 1662-1668.

151. Ichikawa T, Horie-Inoue K, Ikeda K, Blumberg B, Inoue S. Steroid and xenobiotic receptor SXR mediates vitamin K2-activated transcription of extracellular matrix-related genes and collagen accumulation in osteoblastic cells. *J Biol Chem,* **2006***; 281(25)*; 16927-16934.

152. Islam KN, Devaraj S, Jialal I. alpha-Tocopherol enrichment of monocytes decreases agonist-induced adhesion to human endothelial cells. *Circulation,* **1998***; 98(21)*; 2255-2261.

153. Ito Y, Suzuki K, Ishii J, Hishida H, Tamakoshi A, Hamajima N, Aoki K. A population-based follow-up study on mortality from cancer or cardiovascular disease and serum carotenoids, retinol and tocopherols in Japanese inhabitants. *Asian Pac J Cancer Prev,* **2006***; 7(4)*; 533-546.

154. Izgüt-Uysal VN, Tan R, Bülbül M, Derin N. Effect of stress-induced lipid peroxidation on functions of rat peritoneal macrophages. *Cell Biol Int,* **2004***; 28(7)*; 517-521.

155. Jakubowski H. The pathophysiological hypothesis of homocysteine thiolactone-mediated vascular disease. *J Physiol Pharmacol,* **2008***; 59 Suppl 9*; 155-167.

156. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature,* **1996***; 383(6602)*; 728-731.

157. Jiang Q, Ames BN. Gamma-tocopherol, but not alpha-tocopherol, decreases proinflammatory eicosanoids and inflammation damage in rats. *FASEB J,* **2003***; 17(8)*; 816-822.

158. Jiang Q, Elson-Schwab I, Courtemanche C, Ames BN. gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. *Proc Natl Acad Sci U.S.A.,* **2000***; 97(21)*; 11494-11499.

159. Jiang Q, Freiser H, Wood KV, Yin X. Identification and quantitation of novel vitamin E metabolites, sulfated long-chain carboxychromanols, in human A549 cells and in rats. *J Lipid Res,* **2007***; 48(5)*; 1221-1230.

160. Jiang Q, Yin X, Lill MA, Danielson ML, Freiser H, Huang J. Long-chain carboxychromanols, metabolites of vitamin E, are potent inhibitors of cyclooxygenases. *Proc Natl Acad Sci U.S.A.,* **2008***; 105(51)*; 20464-20469.

161. Jiang Z, Yin X, Jiang Q. Natural Forms of Vitamin E and 13'-Carboxychromanol, a Long-Chain Vitamin E Metabolite, Inhibit Leukotriene Generation from Stimulated Neutrophils by Blocking Calcium Influx and Suppressing 5-Lipoxygenase Activity, Respectively. *J Immunol,* **2011***; 186(2)*; 1173-1179.

162. Johnson CH, Slanar O, Krausz KW, Kang DW, Patterson AD, Kim J, Luecke H, Gonzalez FJ, Idle JR. Novel metabolites and roles for -tocopherol in humans and mice discovered by mass spectrometry-based metabolomics. *Am J Clin Nutr,* **2012***; 96(4)*; 818-830.

163. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med,* **2003***; 9(2)*; 213-219.

164. Kalsotra A, Strobel HW. Cytochrome P450 4F subfamily: at the crossroads of eicosanoid and drug metabolism. *Pharmacol Ther,* **2006***; 112(3)*; 589-611.

165. Kalupov T, Brillard-Bourdet M, Dadé S, Serrano H, Wartelle J, Guyot N, Juliano L, Moreau T, Belaaouaj A, Gauthier F. Structural characterization of mouse neutrophil serine proteases and identification of their substrate specificities: relevance to mouse models of human inflammatory diseases. *J Biol Chem,* **2009***; 284(49)*; 34084-34091.

166. Kandutsch AA, Chen HW, Heiniger HJ. Biological activity of some oxygenated sterols. *Science,* **1978***; 201(4355)*; 498-501.

167. Kardinaal AF, Kok FJ, Ringstad J, Gomez-Aracena J, Mazaev VP, Kohlmeier L, Martin BC, Aro A, Kark JD, Delgado-Rodriguez M. Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC Study. *Lancet,* **1993***; 342(8884)*; 1379-1384.

168. Keaney JF, Gaziano JM, Xu A, Frei B, Curran-Celentano J, Shwaery GT, Loscalzo J, Vita JA. Low-dose alpha-tocopherol improves and high-dose alpha-tocopherol worsens endothelial vasodilator function in cholesterol-fed rabbits. *J Clin Invest,* **1994***; 93(2)*; 844-851.

169. Keli SO, Hertog MG, Feskens EJ, Kromhout D. Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study. *Arch Intern Med,* **1996***; 156(6)*; 637-642.

170. Kikuta Y, Kusunose E, Kusunose M. Characterization of human liver leukotriene B(4) omega-hydroxylase P450 (CYP4F2). *J Biochem,* **2000***; 127(6)*; 1047-1052.

171. Kim S, Lee E, Kim SH, Lee S, Lim S. Comparison of three tocopherol analogs as an inhibitor of production of proinflammatory mediators in macrophages. *J Pharmacol Sci,* **2012***; 118(2)*; 237-244.

172. Kiyose C, Muramatsu R, Fujiyama-Fujiwara Y, Ueda T, Igarashi O. Biodiscrimination of alpha-tocopherol stereoisomers during intestinal absorption. *Lipids,* **1995***; 30(11)*; 1015-1018.

173. Kiyose C, Muramatsu R, Kameyama Y, Ueda T, Igarashi O. Biodiscrimination of alpha-tocopherol stereoisomers in humans after oral administration. *Am J Clin Nutr,* **1997***; 65(3)*; 785-789.

174. Kiyose C, Saito H, Kaneko K, Hamamura K, Tomioka M, Ueda T, Igarashi O. Alpha-tocopherol affects the urinary and biliary excretion of 2,7,8-trimethyl-2 (2'-carboxyethyl)-6-hydroxychroman, gamma-tocopherol metabolite, in rats. *Lipids,* **2001***; 36(5)*; 467-472.

175. Klein EA, Thompson IM. Chemoprevention of prostate cancer: an updated view. *World J Urol,* **2012***; 30(2)*; 189-194.

176. Klipstein-Grobusch K, Geleijnse JM, den Breeijen JH, Boeing H, Hofman A, Grobbee DE, Witteman JC. Dietary antioxidants and risk of myocardial infarction in the elderly: the Rotterdam Study. *Am J Clin Nutr,* **1999***; 69(2)*; 261-266.

177. Kluth D, Landes N, Pfluger P, Müller-Schmehl K, Weiss K, Bumke-Vogt C, Ristow M, Brigelius-Flohé R. Modulation of Cyp3a11 mRNA expression by alpha-tocopherol but not gamma-tocotrienol in mice. *Free Radic Biol Med,* **2005***; 38(4)*; 507-514.

178. Knekt P, Reunanen A, Järvinen R, Seppänen R, Heliövaara M, Aromaa A. Antioxidant vitamin intake and coronary mortality in a longitudinal population study. *Am J Epidemiol,* **1994***; 139(12)*; 1180-1189.

179. Knekt P, Ritz J, Pereira MA, O'Reilly EJ, Augustsson K, Fraser GE, Goldbourt U, Heitmann BL, Hallmans G, Liu S, Pietinen P, Spiegelman D, Stevens J, Virtamo J, Willett WC, Rimm EB, Ascherio A. Antioxidant vitamins and coronary heart disease risk: a pooled analysis of 9 cohorts. *Am J Clin Nutr,* **2004***; 80(6)*; 1508-1520.

180. Koga T, Kwan P, Zubik L, Ameho C, Smith D, Meydani M. Vitamin E supplementation suppresses macrophage accumulation and endothelial cell expression of adhesion molecules in the aorta of hypercholesterolemic rabbits. *Atherosclerosis,* **2004***; 176(2)*; 265-272.

181. Kohlmeier M. Nutrient Metabolism. Academic Press, Spain, **2003**.

182. Kok FJ, de Bruijn AM, Vermeeren R, Hofman A, van Laar A, Bruin M de, Hermus RJ, Valkenburg HA. Serum selenium, vitamin antioxidants, and cardiovascular mortality: a 9-year follow-up study in the Netherlands. *Am J Clin Nutr,* **1987***; 45(2)*; 462-468.

183. Kontush A, Finckh B, Karten B, Kohlschütter A, Beisiegel U. Antioxidant and prooxidant activity of alpha-tocopherol in human plasma and low density lipoprotein. *J Lipid Res,* **1996***; 37(7)*; 1436-1448.

184. Kostner K, Hornykewycz S, Yang P, Neunteufl T, Glogar D, Weidinger F, Maurer G, Huber K. Is oxidative stress causally linked to unstable angina pectoris? A study in 100 CAD patients and matched controls. *Cardiovasc Res,* **1997***; 36(3)*; 330-336.

185. Kruk J, Pisarski A, Szymańska R. Novel vitamin E forms in leaves of Kalanchoe daigremontiana and Phaseolus coccineus. *J Plant Physiol,* **2011***; 168(17)*; 2021-2027.

186. Kullo IJ, Gau GT, Tajik AJ. Novel risk factors for atherosclerosis. *Mayo Clin Proc,* **2000***; 75(4)*; 369-380.

187. Kushi LH, Folsom AR, Prineas RJ, Mink PJ, Wu Y, Bostick RM. Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. *N Engl J Med,* **1996***; 334(18)*; 1156-1162.

188. Landes N, Pfluger P, Kluth D, Birringer M, Rühl R, Böl G, Glatt H, Brigelius-Flohé R. Vitamin E activates gene expression via the pregnane X receptor. *Biochem Pharmacol,* **2003***; 65(2)*; 269-273.

189. Landrier J, Gouranton E, El Yazidi C, Malezet C, Balaguer P, Borel P, Amiot M. Adiponectin expression is induced by vitamin E via a peroxisome proliferator-activated receptor gamma-dependent mechanism. *Endocrinology,* **2009***; 150(12)*; 5318-5325.

190. Lasker JM, Chen WB, Wolf I, Bloswick BP, Wilson PD, Powell PK. Formation of 20-hydroxyeicosatetraenoic acid, a vasoactive and natriuretic eicosanoid, in human kidney. Role of Cyp4F2 and Cyp4A11. *J Biol Chem,* **2000***; 275(6)*; 4118-4126.

191. Lattin J, Zidar DA, Schroder K, Kellie S, Hume DA, Sweet MJ. G-protein-coupled receptor expression, function, and signaling in macrophages. *J Leukoc Biol,* **2007***; 82(1)*; 16-32.

192. Lavaud A, Richomme P, Litaudon M, Andriantsitohaina R, Guilet D. Antiangiogenic tocotrienol derivatives from Garcinia amplexicaulis. *J Nat Prod,* **2013***; 76(12)*; 2246-2252.

193. Law MR, Morris JK. By how much does fruit and vegetable consumption reduce the risk of ischaemic heart disease? *Eur J Clin Nutr,* **1998***; 52(8)*; 549-556.

194. Lebold KM, Ang A, Traber MG, Arab L. Urinary α-carboxyethyl hydroxychroman can be used as a predictor of α-tocopherol adequacy, as demonstrated in the Energetics Study. *Am J Clin Nutr,* **2012***; 96(4)*; 801-809.

195. Lee I, Cook NR, Gaziano JM, Gordon D, Ridker PM, Manson JE, Hennekens CH, Buring JE. Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial. *JAMA,* **2005***; 294(1)*; 56-65.

196. Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest,* **1998***; 102(5)*; 1016-1023.

197. Lemaire-Ewing S, Desrumaux C, Néel D, Lagrost L. Vitamin E transport, membrane incorporation and cell metabolism: Is alpha-tocopherol in lipid rafts an oar in the lifeboat? *Mol Nutr Food Res,* **2010***; 54(5)*; 631-640.

198. Leppälä JM, Virtamo J, Fogelholm R, Huttunen JK, Albanes D, Taylor PR, Heinonen OP. Controlled trial of alpha-tocopherol and beta-carotene supplements on stroke incidence and mortality in male smokers. *Arterioscler Thromb Vasc Biol,* **2000***; 20(1)*; 230-235.

199. Leth T, Sondergaard H. Biological activity of vitamin E compounds and natural materials by the resorption-gestation test, and chemical determination of the vitamin E activity in foods and feeds. **1977**.

200. Li D, Saldeen T, Mehta JL. Effects of alpha-tocopherol on ox-LDL-mediated degradation of IkappaB and apoptosis in cultured human coronary artery endothelial cells. *J Cardiovasc Pharmacol,* **2000***; 36(3)*; 297-301.

201. Li W, Hellsten A, Jacobsson LS, Blomqvist HM, Olsson AG, Yuan X. Alpha-tocopherol and astaxanthin decrease macrophage infiltration, apoptosis and vulnerability in atheroma of hyperlipidaemic rabbits. *J Mol Cell Cardiol,* **2004***; 37(5)*; 969-978.

202. Libby P. Inflammation in atherosclerosis. *Nature,* **2002***; 420(6917)*; 868-874.

203. Libinaki R, Tesanovic S, Heal A, Nikolovski B, Vinh A, Widdop RE, Gaspari TA, Devaraj S, Ogru E. Effect of tocopheryl phosphate on key biomarkers of inflammation: Implication in the reduction of atherosclerosis progression in a hypercholesterolaemic rabbit model. *Clin Exp Pharmacol Physiol,* **2010***; 37(5-6)*; 587-592.

204. Lloret A, Badía M, Mora NJ, Pallardó FV, Alonso M, Viña J. Vitamin E paradox in Alzheimer's disease: it does not prevent loss of cognition and may even be detrimental. *J Alzheimers Dis,* **2009***; 17(1)*; 143-149.

205. Lonn E, Bosch J, Yusuf S, Sheridan P, Pogue J, Arnold, J Malcolm O, Ross C, Arnold A, Sleight P, Probstfield J, Dagenais GR. Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA,* **2005***; 293(11)*; 1338-1347.

206. Losonczy KG, Harris TB, Havlik RJ. Vitamin E and vitamin C supplement use and risk of all-cause and coronary heart disease mortality in older persons: the Established Populations for Epidemiologic Studies of the Elderly. *Am J Clin Nutr,* **1996***; 64(2)*; 190-196.

207. Lund EG, Kerr TA, Sakai J, Li WP, Russell DW. cDNA cloning of mouse and human cholesterol 25-hydroxylases, polytopic membrane proteins that synthesize a potent oxysterol regulator of lipid metabolism. *J Biol Chem,* **1998***; 273(51)*; 34316-34327.

208. Lyons NM, Woods JA, O'Brien NM. alpha-Tocopherol, but not gamma-tocopherol inhibits 7 beta-hydroxycholesterol-induced apoptosis in human U937 cells. *Free Radic Res,* **2001***; 35(3)*; 329-339.

209. Marchioli R. Antioxidant vitamins and prevention of cardiovascular disease: laboratory, epidemiological and clinical trial data. *Pharmacol Res,* **1999***; 40(3)*; 227-238.

210. Mark SD, Wang W, Fraumeni JF, Li JY, Taylor PR, Wang GQ, Guo W, Dawsey SM, Li B, Blot WJ. Lowered risks of hypertension and cerebrovascular disease after vitamin/mineral supplementation: the Linxian Nutrition Intervention Trial. *Am J Epidemiol,* **1996***; 143(7)*; 658-664.

211. Marquardt D, Williams JA, Kučerka N, Atkinson J, Wassall SR, Katsaras J, Harroun TA. Tocopherol Activity Correlates with Its Location in a Membrane: A New Perspective on the Antioxidant Vitamin E. *J Am Chem Soc,* **2013***; 135(20)*; 7523-7533.

212. Masuyama H, Suwaki N, Tateishi Y, Nakatsukasa H, Segawa T, Hiramatsu Y. The pregnane X receptor regulates gene expression in a ligand- and promoter-selective fashion. *Mol Endocrinol,* **2005***; 19(5)*; 1170-1180.

213. May P. The low-density lipoprotein receptor-related protein 1 in inflammation. *Curr Opin Lipidol,* **2013***; 24(2)*; 134-137.

214. Mazzini F, Betti M, Netscher T, Galli F, Salvadori P. Configuration of the vitamin E analogue garcinoic acid extracted from garcinia kola seeds. *Chirality,* **2009***; 21(5)*; 519-524.

215. McCary CA, Yoon Y, Panagabko C, Cho W, Atkinson J, Cook-Mills JM. Vitamin E isoforms directly bind PKCα and differentially regulate activation of PKCα. *Biochem J,* **2012***; 441(1)*; 189-198.

216. McLaren JE, Michael DR, Guschina IA, Harwood JL, Ramji DP. Eicosapentaenoic acid and docosahexaenoic acid regulate modified LDL uptake and macropinocytosis in human macrophages. *Lipids,* **2011***; 46(11)*; 1053-1061.

217. Mene-Saffrane L, Jones AD, DellaPenna D. Plastochromanol-8 and tocopherols are essential lipid-soluble antioxidants during seed desiccation and quiescence in Arabidopsis. *Proc Natl Acad Sci,* **2010***; 107(41)*; 17815-17820.

218. Meraji S, Abuja PM, Hayn M, Kostner GM, Morris R, Oraii S, Tatzber F, Wonisch W, Zechner R, Gey KF. Relationship between classic risk factors, plasma antioxidants and indicators of oxidant stress in angina pectoris (AP) in Tehran. *Atherosclerosis,* **2000***; 150(2)*; 403-412.

219. Meydani M, Kwan P, Band M, Knight A, Guo W, Goutis J, Ordovas J. Long-term vitamin E supplementation reduces atherosclerosis and mortality in Ldlr-/-mice, but not when fed Western style diet. *Atherosclerosis,* **2014**.

220. Miller ER. Meta-Analysis: High-Dosage Vitamin E Supplementation May Increase All-Cause Mortality. *Ann Intern Med,* **2005***; 142(1)*; 37.

221. Monajemi H, Arkenbout EK, Pannekoek H. Gene expression in atherogenesis. *Thromb Haemost,* **2001***; 86(1)*; 404-412.

222. Mosig S, Rennert K, Krause S, Kzhyshkowska J, Neunübel K, Heller R, Funke H. Different functions of monocyte subsets in familial hypercholesterolemia: potential function of CD14+ CD16+ monocytes in detoxification of oxidized LDL. *FASEB J,* **2009***; 23(3)*; 866-874.

223. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol,* **2008***; 8(12)*; 958-969.

224. Munteanu A, Taddei M, Tamburini I, Bergamini E, Azzi A, Zingg J. Antagonistic effects of oxidized low density lipoprotein and alpha-tocopherol on CD36 scavenger receptor expression in monocytes: involvement of protein kinase B and peroxisome proliferator-activated receptor-gamma. *J Biol Chem,* **2006***; 281(10)*; 6489-6497.

225. Muntwyler J, Hennekens CH, Manson JE, Buring JE, Gaziano JMichael. Vitamin supplement use in a low-risk population of US male physicians and subsequent cardiovascular mortality. *Arch Intern Med,* **2002***; 162(13)*; 1472-1476.

226. Murakami M. Lipid mediators in life science. *Exp Anim,* **2011***; 60(1)*; 7-20.

227. Murray CJL, Frenk J. Health metrics and evaluation: strengthening the science. *The Lancet,* **2008***; 371(9619)*; 1191-1199.

228. Mustacich DJ, Gohil K, Bruno RS, Yan M, Leonard SW, Ho E, Cross CE, Traber MG. Alpha-tocopherol modulates genes involved in hepatic xenobiotic pathways in mice. *J Nutr Biochem,* **2009***; 20(6)*; 469-476.

229. Mustacich DJ, Leonard SW, Devereaux MW, Sokol RJ, Traber MG. Alpha-tocopherol regulation of hepatic cytochrome P450s and ABC transporters in rats. *Free Radic Biol Med,* **2006***; 41(7)*; 1069-1078.

230. Mustacich DJ, Leonard SW, Patel NK, Traber MG. Alpha-tocopherol beta-oxidation localized to rat liver mitochondria. *Free Radic Biol Med,* **2010***; 48(1)*; 73-81.

231. Mustacich DJ, Shields J, Horton RA, Brown MK, Reed DJ. Biliary secretion of alpha-tocopherol and the role of the mdr2 P-glycoprotein in rats and mice. *Arch Biochem Biophys,* **1998***; 350(2)*; 183-192.

232. Nakamura YK, Omaye ST. Alpha-tocopherol modulates human umbilical vein endothelial cell expression of Cu/Zn superoxide dismutase and catalase and lipid peroxidation. *Nutr Res,* **2008***; 28(10)*; 671-680.

233. Narushima K, Takada T, Yamanashi Y, Suzuki H. Niemann-pick C1-like 1 mediates alpha-tocopherol transport. *Mol Pharmacol,* **2008***; 74(1)*; 42-49.

234. Negis Y, Meydani M, Zingg J, Azzi A. Molecular mechanism of alpha-tocopheryl-phosphate transport across the cell membrane. *Biochem Biophys Res Commun,* **2007***; 359(2)*; 348-353.

235. Neuzil J, Kagedal K, Andera L, Weber C, Brunk UT. Vitamin E analogs: A new class of multiple action agents with anti-neoplastic and anti-atherogenic activity. *Apoptosis,* **2002***(7)*; 179-187.

236. Newby AC. Metalloproteinase Expression in Monocytes and Macrophages and its Relationship to Atherosclerotic Plaque Instability. *Arterioscler Thromb Vasc Biol,* **2008***; 28(12)*; 2108-2114.

237. Ng L, Ko H. Comparative effects of tocotrienol-rich fraction, α-tocopherol and α-tocopheryl acetate on inflammatory mediators and nuclear factor kappa B expression in mouse peritoneal macrophages. *Food Chem,* **2012***; 134(2)*; 920-925.

238. Nguyen A, Tao H, Metrione M, Hajri T. Very low density lipoprotein receptor expression is a determinant factor in adipose tissue inflammation and adipocyte-macrophage interaction. *J Biol Chem,* **2013**.

239. Ni J, Wen X, Yao J, Chang H, Yin Y, Zhang M, Xie S, Chen M, Simons B, Chang P, Di Sant'Agnese A, Messing EM, Yeh S. Tocopherol-associated protein suppresses prostate cancer cell growth by inhibition of the phosphoinositide 3-kinase pathway. *Cancer Res,* **2005***; 65(21)*; 9807-9816.

240. Nobata Y, Urakaze M, Temaru R, Sato A, Nakamura N, Yamazaki K, Kishida M, Takata M, Kobayashi M. alpha-Tocopherol Inhibits IL-8 synthesis induced by thrombin and high glucose in endothelial cells. *Horm Metab Res,* **2002***; 34(2)*; 49-54.

241. Noguchi N, Hanyu R, Nonaka A, Okimoto Y, Kodama T. Inhibition of THP-1 cell adhesion to endothelial cells by alpha-tocopherol and alpha-tocotrienol is dependent on intracellular concentration of the antioxidants. *Free Radic Biol Med,* **2003***; 34(12)*; 1614-1620.

242. Nojiri S, Daida H, Mokuno H, Iwama Y, Mae K, Ushio F, Ueki T. Association of serum antioxidant capacity with coronary artery disease in middle-aged men. *Jpn Heart J,* **2001***; 42(6)*; 677-690.

243. Olejnik D, Gogolewski M, Nogala-Kaiucka M. Isolation and some properties of plastochromanol-8. last checked 30.01.2014; https://www.google.de/#q=isolation+and+some+properties+of+plastochromanol-8.

244. Oliveira FLC, Patin RV, Escrivão MAMS. Atherosclerosis prevention and treatment in children and adolescents. *Expert Rev Cardiovasc Ther,* **2010***; 8(4)*; 513-528.

245. Oram JF, Vaughan AM, Stocker R. ATP-binding cassette transporter A1 mediates cellular secretion of alpha-tocopherol. *J Biol Chem,* **2001***; 276(43)*; 39898-39902.

246. Ouahchi K, Arita M, Kayden H, Hentati F, Ben Hamida M, Sokol R, Arai H, Inoue K, Mandel JL, Koenig M. Ataxia with isolated vitamin E deficiency is caused by mutations in the alpha-tocopherol transfer protein. *Nat Genet,* **1995***; 9(2)*; 141-145.

247. Ozer NK, Sirikçi O, Taha S, San T, Moser U, Azzi A. Effect of vitamin E and probucol on dietary cholesterol-induced atherosclerosis in rabbits. *Free Radic Biol Med,* **1998***; 24(2)*; 226-233.

248. Panin G, Strumia R, Ursini F. Topical alpha-tocopherol acetate in the bulk phase: eight years of experience in skin treatment. *Ann N Y Acad Sci,* **2004***; 1031*; 443-447.

249. Pant S, Deshmukh A, GuruMurthy GS, Pothineni NV, Watts TE, Romeo F, Mehta JL. Inflammation and Atherosclerosis--Revisited. *J Cardiovasc Pharmacol Ther,* **2013**.

250. Parker RS, McCormick CC. Selective accumulation of alpha-tocopherol in Drosophila is associated with cytochrome P450 tocopherol-omega-hydroxylase activity but not alpha-tocopherol transfer protein. *Biochem Biophys Res Commun,* **2005***; 338(3)*; 1537-1541.

251. Parker RS, Sontag TJ, Swanson JE. Cytochrome P4503A-dependent metabolism of tocopherols and inhibition by sesamin. *Biochem Biophys Res Commun,* **2000***; 277(3)*; 531-534.

252. Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG, Glass CK. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature,* **2005***; 437(7059)*; 759-763.

253. Pearce BC, Parker RA, Deason ME, Qureshi AA, Wright JJ. Hypocholesterolemic activity of synthetic and natural tocotrienols. *J Med Chem,* **1992***; 35(20)*; 3595-3606.

254. Pearson P, Lewis SA, Britton J, Young IS, Fogarty A. The pro-oxidant activity of high-dose vitamin E supplements in vivo. *BioDrugs,* **2006***; 20(5)*; 271-273.

255. Poirier J, Cockell KA, Ratnayake, W M Nimal, Scoggan KA, Hidiroglou N, Gagnon C, Rocheleau H, Gruber H, Griffin P, Madère R, Trick K, Kubow S. Antioxidant Supplements Improve Profiles of Hepatic Oxysterols and Plasma Lipids in Butter-fed Hamsters. *Nutr Metab Insights,* **2010***; 3*; 1-14.

256. Pramfalk C, Eriksson M, Parini P. Cholesteryl esters and ACAT. *Eur J Lipid Sci Technol,* **2012***; 114(6)*; 624-633.

257. Praticò D, Tangirala RK, Rader DJ, Rokach J, FitzGerald GA. Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in ApoE-deficient mice. *Nat Med,* **1998***; 4(10)*; 1189-1192.

258. Qureshi AA, Mo H, Packer L, Peterson DM. Isolation and identification of novel tocotrienols from rice bran with hypocholesterolemic, antioxidant, and antitumor properties. *J Agric Food Chem,* **2000***; 48(8)*; 3130-3140.

259. Qureshi AA, Peterson DM, Hasler-Rapacz JO, Rapacz J. Novel tocotrienols of rice bran suppress cholesterogenesis in hereditary hypercholesterolemic swine. *J Nutr,* **2001a***; 131(2)*; 223-230.

260. Qureshi AA, Salser WA, Parmar R, Emeson EE. Novel tocotrienols of rice bran inhibit atherosclerotic lesions in C57BL/6 ApoE-deficient mice. *J Nutr,* **2001b***; 131(10)*; 2606-2618.

261. Raal FJ. Pathogenesis and Management of the Dyslipidemia of the Metabolic Syndrome. *Metabolic Syndrome and Related Disorders,* **2009***; 7(2)*; 83-88.

262. Rabkin SW, Lodhia P, Lodha P. Stearic acid-induced cardiac lipotoxicity is independent of cellular lipid and is mitigated by the fatty acids oleic and capric acid but not by the PPAR agonist troglitazone. *Exp Physiol,* **2009***; 94(8)*; 877-887.

263. Rajasekhar D, Srinivasa Rao PVLN, Latheef SAA, Saibaba KSS, Subramanyam G. Association of serum antioxidants and risk of coronary heart disease in South Indian population. *Indian J Med Sci,* **2004***; 58(11)*; 465-471.

264. Rapola JM, Virtamo J, Ripatti S, Huttunen JK, Albanes D, Taylor PR, Heinonen OP. Randomised trial of alpha-tocopherol and beta-carotene supplements on incidence of major coronary events in men with previous myocardial infarction. *Lancet,* **1997***; 349(9067)*; 1715-1720.

265. Reboul E, Klein A, Bietrix F, Gleize B, Malezet-Desmoulins C, Schneider M, Margotat A, Lagrost L, Collet X, Borel P. Scavenger receptor class B type I (SR-BI) is involved in vitamin E transport across the enterocyte. *J Biol Chem,* **2006***; 281(8)*; 4739-4745.

266. Ricardo SD, van Goor H, Eddy AA. Macrophage diversity in renal injury and repair. *J Clin Invest,* **2008***; 118(11)*; 3522-3530.

267. Ricciarelli R, Tasinato A, Clément S, Ozer NK, Boscoboinik D, Azzi A. alpha-Tocopherol specifically inactivates cellular protein kinase C alpha by changing its phosphorylation state. *Biochem J,* **1998***; 334 (Pt 1)*; 243-249.

268. Ricciarelli R, Zingg JM, Azzi A. Vitamin E reduces the uptake of oxidized LDL by inhibiting CD36 scavenger receptor expression in cultured aortic smooth muscle cells. *Circulation,* **2000***; 102(1)*; 82-87.

269. Richelle M, Enslen M, Hager C, Groux M, Tavazzi I, Godin J, Berger A, Métairon S, Quaile S, Piguet-Welsch C, Sagalowicz L, Green H, Fay LB. Both free and esterified plant sterols reduce cholesterol absorption and the bioavailability of beta-carotene and alpha-tocopherol in normocholesterolemic humans. *Am J Clin Nutr,* **2004***; 80(1)*; 171-177.

270. Riemersma RA, Wood DA, Macintyre CC, Elton RA, Gey KF, Oliver MF. Risk of angina pectoris and plasma concentrations of vitamins A, C, and E and carotene. *Lancet,* **1991***; 337(8732)*; 1-5.

271. Rigotti A. Absorption, transport, and tissue delivery of vitamin E. *Mol Aspects Med,* **2007***; 28(5-6)*; 423-436.

272. Rimbach G, Fischer A, Stoecklin E, Barella L. Modulation of hepatic gene expression by alpha-tocopherol in cultured cells and in vivo. *Ann N Y Acad Sci,* **2004***; 1031*; 102-108.

273. Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk of coronary heart disease in men. *N Engl J Med,* **1993***; 328(20)*; 1450-1456.

274. Robbins CS, Hilgendorf I, Weber GF, Theurl I, Iwamoto Y, Figueiredo J, Gorbatov R, Sukhova GK, Gerhardt LMS, Smyth D, Zavitz CCJ, Shikatani EA, Parsons M, van Rooijen N, Lin HY, Husain M, Libby P, Nahrendorf M, Weissleder R, Swirski FK. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat Med,* **2013***; 19(9)*; 1166-1172.

275. Roberts LJ, Oates JA, Linton MF, Fazio S, Meador BP, Gross MD, Shyr Y, Morrow JD. The relationship between dose of vitamin E and suppression of oxidative stress in humans. *Free Radic Biol Med,* **2007***; 43(10)*; 1388-1393.

276. Rode S, Rubic T, Lorenz RL. alpha-Tocopherol disturbs macrophage LXRalpha regulation of ABCA1/G1 and cholesterol handling. *Biochem Biophys Res Commun,* **2008***; 369(3)*; 868-872.

277. Rodrigo R, Guichard C, Charles R. Clinical pharmacology and therapeutic use of antioxidant vitamins. *Fundam Clin Pharmacol,* **2007***; 21(2)*; 111-127.

278. Rogi T, Tomimori N, Ono Y, Kiso Y. The mechanism underlying the synergetic hypocholesterolemic effect of sesamin and α-tocopherol in rats fed a high-cholesterol diet. *J Pharmacol Sci,* **2011***; 115(3)*; 408-416.

279. Ross RK, Yuan JM, Henderson BE, Park J, Gao YT, Yu MC. Prospective evaluation of dietary and other predictors of fatal stroke in Shanghai, China. *Circulation,* **1997***; 96(1)*; 50-55.

280. Royer M, Lemaire-Ewing S, Desrumaux C, Monier S, Pais de Barros, Jean-Paul, Athias A, Néel D, Lagrost L. 7-ketocholesterol incorporation into sphingolipid/cholesterol-enriched (lipid raft) domains is impaired by vitamin E: a specific role for alpha-tocopherol with consequences on cell death. *J Biol Chem,* **2009***; 284(23)*; 15826-15834.

281. Rühli FJ, Chhem RK, Böni T. Diagnostic paleoradiology of mummified tissue: interpretation and pitfalls. *Can Assoc Radiol J,* **2004***; 55(4)*; 218-227.

282. Russell DW. Nuclear orphan receptors control cholesterol catabolism. *Cell,* **1999***; 97(5)*; 539-542.

283. Sahyoun NR, Jacques PF, Russell RM. Carotenoids, vitamins C and E, and mortality in an elderly population. *Am J Epidemiol,* **1996***; 144(5)*; 501-511.

284. Salonen JT, Nyyssönen K, Salonen R, Lakka HM, Kaikkonen J, Porkkala-Sarataho E, Voutilainen S, Lakka TA, Rissanen T, Leskinen L, Tuomainen TP, Valkonen VP, Ristonmaa U, Poulsen HE. Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study: a randomized trial of the effect of vitamins E and C on 3-year progression of carotid atherosclerosis. *J Intern Med,* **2000***; 248(5)*; 377-386.

285. Salonen JT, Salonen R, Penttilä I, Herranen J, Jauhiainen M, Kantola M, Lappeteläinen R, Mäenpää PH, Alfthan G, Puska P. Serum fatty acids, apolipoproteins, selenium and vitamin antioxidants and the risk of death from coronary artery disease. *Am J Cardiol,* **1985***; 56(4)*; 226-231.

286. Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, Woodbury P, Growdon J, Cotman CW, Pfeiffer E, Schneider LS, Thal LJ. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N Engl J Med,* **1997***; 336(17)*; 1216-1222.

287. Satchell L, Leake DS. Oxidation of low-density lipoprotein by iron at lysosomal pH: implications for atherosclerosis. *Biochemistry,* **2012***; 51(18)*; 3767-3775.

288. Schnoor M, Buers I, Sietmann A, Brodde MF, Hofnagel O, Robenek H, Lorkowski S. Efficient non-viral transfection of THP-1 cells. *J Immunol Methods,* **2009***; 344(2)*; 109-115.

289. Schnoor M, Cullen P, Lorkowski J, Stolle K, Robenek H, Troyer D, Rauterberg J, Lorkowski S. Production of type VI collagen by human macrophages: a new dimension in macrophage functional heterogeneity. *J Immunol,* **2008***; 180(8)*; 5707-5719.

290. Schrijvers DM, De Meyer, Guido R Y, Herman AG, Martinet W. Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque progression and stability. *Cardiovasc Res,* **2007***; 73(3)*; 470-480.

291. Schroepfer GJ. Oxysterols: modulators of cholesterol metabolism and other processes. *Physiol Rev,* **2000***; 80(1)*; 361-554.

292. Schuelke M, Elsner A, Finckh B, Kohlschütter A, Hübner C, Brigelius-Flohé R. Urinary alpha-tocopherol metabolites in alpha-tocopherol transfer protein-deficient patients. *J Lipid Res,* **2000***; 41(10)*; 1543-1551.

293. Schultz M, Leist M, Petrzika M, Gassmann B, Brigelius-Flohé R. Novel urinary metabolite of alpha-tocopherol, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman, as an indicator of an adequate vitamin E supply? *Am J Clin Nutr,* **1995***; 62(6 Suppl)*; 1527S-1534S.

294. Schwartz CJ, Valente AJ, Sprague EA, Kelley JL, Nerem RM. The pathogenesis of atherosclerosis: an overview. *Clin Cardiol,* **1991***; 14(2 Suppl 1)*; I1-16.

295. Schwartz JL. The dual roles of nutrients as antioxidants and prooxidants: their effects on tumor cell growth. *J Nutr,* **1996***; 126(4 Suppl)*; 1221S-7S.

296. Sen CK, Khanna S, Roy S. Tocotrienols in health and disease: the other half of the natural vitamin E family. *Mol Aspects Med,* **2007***; 28(5-6)*; 692-728.

297. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, McDonald-Smith GP, Gao H, Hennessy L, Finnerty CC, López CM, Honari S, Moore EE, Minei JP, Cuschieri J, Bankey PE, Johnson JL, Sperry J, Nathens AB, Billiar TR, West MA, Jeschke MG, Klein MB, Gamelli RL, Gibran NS, Brownstein BH, Miller-Graziano C, Calvano SE, Mason PH, Cobb JP, Rahme LG, Lowry SF, Maier RV, Moldawer LL, Herndon DN, Davis RW, Xiao W, Tompkins RG. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U.S.A.,* **2013***; 110(9)*; 3507-3512.

298. Sesso HD, Buring JE, Christen WG, Kurth T, Belanger C, MacFadyen J, Bubes V, Manson JE, Glynn RJ, Gaziano JM. Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial. *JAMA,* **2008***; 300(18)*; 2123-2133.

299. Shekelle PG, Morton SC, Jungvig LK, Udani J, Spar M, Tu W, J Suttorp M, Coulter I, Newberry SJ, Hardy M. Effect of supplemental vitamin E for the prevention and treatment of cardiovascular disease. *J Gen Intern Med,* **2004***; 19(4)*; 380-389.

300. Shils ME, Shike M, Ross AC, Caballero B, Cousins RJ. Modern Nutrition. last checked 11.02.2014; http://books.google.de/books?hl=de&lr=&id=S5oCjZZZ1ggC&oi=fnd&pg=PA3&dq=modern+nutrition+in+health+and+disease+10th+edition+Traber&ots=2wQCr0pJyt&sig=o_12COxS38dZAkZw5BMAtLkEECA#v=onepage&q=Traber&f=false.

301. Silverstein RL, Li W, Park YM, Rahaman SO. Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis. *Trans Am Clin Climatol Assoc,* **2010***; 121*; 206-220.

302. Sondergaard E, Dam H. Occurrence of a plastochromanol in linseed oil. **1967**.

303. Sonnen AF-P, Bakirci H, Netscher T, Nau WM. Effect of temperature, cholesterol content, and antioxidant structure on the mobility of vitamin E constituents in biomembrane models studied by laterally diffusion-controlled fluorescence quenching. *J Am Chem Soc,* **2005***; 127(44)*; 15575-15584.

304. Sontag TJ, Parker RS. Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status. *J Biol Chem,* **2002***; 277(28)*; 25290-25296.

305. Spiegelberg BD, Hamm HE. G betagamma binds histone deacetylase 5 (HDAC5) and inhibits its transcriptional co-repression activity. *J Biol Chem,* **2005***; 280(50)*; 41769-41776.

306. Stahl W, Graf P, Brigelius-Flohé R, Wechter W, Sies H. Quantification of the alpha- and gamma-tocopherol metabolites 2,5,7, 8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman and 2,7, 8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman in human serum. *Anal Biochem,* **1999***; 275(2)*; 254-259.

307. Stanton ME, Spear LP. Workshop on the qualitative and quantitative comparability of human and animal developmental neurotoxicity, Work Group I report: comparability of measures of developmental neurotoxicity in humans and laboratory animals. *Neurotoxicol Teratol,* **1990***; 12(3)*; 261-267.

308. Statistisches Bundesamt. Statistisches Jahrbuch. **2012**. last checked28.04.2014.

309. Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet,* **1996***; 347(9004)*; 781-786.

310. Stocker A, Baumann U. Supernatant protein factor in complex with RRR-alpha-tocopherylquinone: a link between oxidized Vitamin E and cholesterol biosynthesis. *J Mol Biol,* **2003***; 332(4)*; 759-765.

311. Stocker A, Zimmer S, Spycher SE, Azzi A. Identification of a novel cytosolic tocopherol-binding protein: structure, specificity, and tissue distribution. *IUBMB Life,* **1999***; 48(1)*; 49-55.

312. Stolle K, Schnoor M, Fuellen G, Spitzer M, Cullen P, Lorkowski S. Cloning, genomic organization, and tissue-specific expression of the RASL11B gene. *Biochim Biophys Acta,* **2007***; 1769(7-8)*; 514-524.

313. Stolle K, Schnoor M, Fuellen G, Spitzer M, Engel T, Spener F, Cullen P, Lorkowski S. Cloning, cellular localization, genomic organization, and tissue-specific expression of the TGFbeta1-inducible SMAP-5 gene. *Gene,* **2005***; 351*; 119-130.

314. Strott CA, Higashi Y. Cholesterol sulfate in human physiology: what's it all about? *J Lipid Res,* **2003***; 44(7)*; 1268-1278.

315. Suarna C, Dean RT, May J, Stocker R. Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of alpha-tocopherol and ascorbate. *Arterioscler Thromb Vasc Biol,* **1995***; 15(10)*; 1616-1624.

316. Sun L, Ye RD. Role of G protein-coupled receptors in inflammation. *Acta Pharmacol Sin,* **2012***; 33(3)*; 342-350.

317. Suzuki YJ, Packer L. Inhibition of NF-kappa B activation by vitamin E derivatives. *Biochem Biophys Res Commun,* **1993***; 193(1)*; 277-283.

318. Swanson JE, Ben RN, Burton GW, Parker RS. Urinary excretion of 2,7, 8-trimethyl-2-(beta-carboxyethyl)-6-hydroxychroman is a major route of elimination of gamma-tocopherol in humans. *J Lipid Res,* **1999***; 40(4)*; 665-671.

319. Sylvester PW, Nachnani A, Shah S, Briski KP. Role of GTP-binding proteins in reversing the antiproliferative effects of tocotrienols in preneoplastic mammary epithelial cells. *Asia Pac J Clin Nutr,* **2002***; 11 Suppl 7*; S452-9.

320. Szabó C, Wu CC, Mitchell JA, Gross SS, Thiemermann C, Vane JR. Platelet-activating factor contributes to the induction of nitric oxide synthase by bacterial lipopolysaccharide. *Circ Res,* **1993***; 73(6)*; 991-999.

321. Talvani A, Machado FS, Santana GC, Klein A, Barcelos L, Silva JS, Teixeira MM. Leukotriene B(4) induces nitric oxide synthesis in Trypanosoma cruzi-infected murine macrophages and mediates resistance to infection. *Infect Immun,* **2002***; 70(8)*; 4247-4253.

322. Tam S, Mok L, Chimini G, Vasa M, Deeley RG. ABCA1 mediates high-affinity uptake of 25-hydroxycholesterol by membrane vesicles and rapid efflux of oxysterol by intact cells. *Am J Physiol Cell Physiol,* **2006***; 291(3)*; C490-502.

323. Tang C, Liu Y, Kessler PS, Vaughan AM, Oram JF. The macrophage cholesterol exporter ABCA1 functions as an anti-inflammatory receptor. *J Biol Chem,* **2009***; 284(47)*; 32336-32343.

324. Terashima K, Shimamura T, Tanabayashi M, Aqil M, Akinniyi JA, Niwa M. Constituents of the Seeds of Garcinia Kola: Two New Antioxidants, Garcinoic Acid and Garcinal. *Heterocycles,* **1997***; 45(8)*; 1559-1566.

325. Teupser D, Thiery J, Seidel D. Alpha-tocopherol down-regulates scavenger receptor activity in macrophages. *Atherosclerosis,* **1999***; 144(1)*; 109-115.

326. Thompson RC, Allam AH, Lombardi GP, Wann LS, Sutherland ML, Sutherland JD, Soliman MA, Frohlich B, Mininberg DT, Monge JM, Vallodolid CM, Cox SL, Abd el-Maksoud G, Badr I, Miyamoto MI, el-Halim Nur el-din, Abd, Narula J, Finch CE, Thomas GS. Atherosclerosis across 4000 years of human history: the Horus study of four ancient populations. *The Lancet,* **2013***; 381(9873)*; 1211-1222.

327. Todd S, Woodward M, Tunstall-Pedoe H, Bolton-Smith C. Dietary antioxidant vitamins and fiber in the etiology of cardiovascular disease and all-causes mortality: results from the Scottish Heart Health Study. *Am J Epidemiol,* **1999***; 150(10)*; 1073-1080.

328. Tomassi G, Silano V. An assessment of the safety of tocopherols as food additives. *Food Chem Toxicol,* **1986***; 24(10-11)*; 1051-1061.

329. Tomita K, Barnes PJ, Adcock IM. The effect of oxidative stress on histone acetylation and IL-8 release. *Biochem Biophys Res Commun,* **2003***; 301(2)*; 572-577.

330. Totta P, Acconcia F, Virgili F, Cassidy A, Weinberg PD, Rimbach G, Marino M. Daidzein-sulfate metabolites affect transcriptional and antiproliferative activities of estrogen receptor-beta in cultured human cancer cells. *J Nutr,* **2005***; 135(11)*; 2687-2693.

331. Traber MG. Mechanisms for the prevention of vitamin E excess. *J lipid Res*, **2013**; 54(9);2295-2306.

332. Traber MG. Vitamin E, nuclear receptors and xenobiotic metabolism. *Arch Biochem Biophys,* **2004***; 423(1)*; 6-11.

333. Traber MG, Kayden HJ. Preferential incorporation of alpha-tocopherol vs gamma-tocopherol in human lipoproteins. *Am J Clin Nutr,* **1989***; 49(3)*; 517-526.

334. Traber MG, Kayden HJ, Green JB, Green MH. Absorption of water-miscible forms of vitamin E in a patient with cholestasis and in thoracic duct-cannulated rats. *Am J Clin Nutr,* **1986***; 44(6)*; 914-923.

335. Traber MG, Labut EM, Leonard SW, Lebold KM. α-Tocopherol injections in rats up-regulate hepatic ABC transporters, but not cytochrome P450 enzymes. *Free Radic Biol Med,* **2011***; 51(11)*; 2031-2040.

336. Traber MG, Packer L. Vitamin E: beyond antioxidant function. *Am J Clin Nutr,* **1995***; 62(6 Suppl)*; 1501S-1509S.

337. Traber MG, Rudel LL, Burton GW, Hughes L, Ingold KU, Kayden HJ. Nascent VLDL from liver perfusions of cynomolgus monkeys are preferentially enriched in RRR-compared

with SRR-alpha-tocopherol: studies using deuterated tocopherols. *J Lipid Res,* **1990***; 31(4)*; 687-694.

338. Traber MG, Sies H. Vitamin E in humans: demand and delivery. *Annu Rev Nutr,* **1996***; 16*; 321-347.
339. Traber MG. Vitamin E Regulatory Mechanisms. *Annu. Rev. Nutr.,* **2007***; 27(1)*; 347-362.
340. Traber MG. Vitamin E and K interactions--a 50-year-old problem. *Nutr Rev,* **2008***; 66(11)*; 624-629.
341. Traber MG. Regulation of xenobiotic metabolism, the only signaling function of alpha-tocopherol? *Mol Nutr Food Res,* **2010***; 54(5)*; 661-668.
342. Traber MG. Mechanisms for the prevention of vitamin E excess. *J Lipid Res,* **2013***; 54(9)*; 2295-2306.
343. Tsimikas S, Miller YI. Oxidative modification of lipoproteins: mechanisms, role in inflammation and potential clinical applications in cardiovascular disease. *Curr Pharm Des,* **2011***; 17(1)*; 27-37.
344. Tucker J, Townsend D. Alpha-tocopherol: roles in prevention and therapy of human disease. *Biomed Pharmacother,* **2005***; 59(7)*; 380-387.
345. Uemura M, Manabe H, Yoshida N, Fujita N, Ochiai J, Matsumoto N, Takagi T, Naito Y, Yoshikawa T. Alpha-tocopherol prevents apoptosis of vascular endothelial cells via a mechanism exceeding that of mere antioxidation. *Eur J Pharmacol,* **2002***; 456(1-3)*; 29-37.
346. Uto-Kondo H, Kiyose C, Ohmori R, Saito H, Taguchi C, Kishimoto Y, Machida N, Hasegawa M, Yoshioka E, Saita E, Hirata Y, Igarashi O, Kondo K. The coantioxidative effects of carboxyethyl-6-hydroxychromans and alpha-Tocopherol. *J Nutr Sci Vitaminol,* **2007***; 53(4)*; 301-305.
347. Valastyan S, Thakur V, Johnson A, Kumar K, Manor D. Novel transcriptional activities of vitamin E: inhibition of cholesterol biosynthesis. *Biochemistry,* **2008***; 47(2)*; 744-752.
348. van Dam B, van Hinsbergh, Victor W M, Stehouwer, Coen D A, Versteilen A, Dekker H, Buytenhek R, Princen HM, Schalkwijk CG. Vitamin E inhibits lipid peroxidation-induced adhesion molecule expression in endothelial cells and decreases soluble cell adhesion molecules in healthy subjects. *Cardiovasc Res,* **2003***; 57(2)*; 563-571.
349. Varga Z, Kosaras E, Komodi E, Katko M, Karpati I, Balla J, Paragh G, Aisa MC, Galli F. Effects of tocopherols and 2,2'-carboxyethyl hydroxychromans on phorbol-ester-stimulated neutrophils. *J Nutr Biochem,* **2008***; 19(5)*; 320-327.
350. Vejux A, Guyot S, Montange T, Riedinger J, Kahn E, Lizard G. Phospholipidosis and down-regulation of the PI3-K/PDK-1/Akt signalling pathway are vitamin E inhibitable events associated with 7-ketocholesterol-induced apoptosis. *J Nutr Biochem,* **2009***; 20(1)*; 45-61.
351. Venugopal SK, Devaraj S, Jialal I. RRR-alpha-tocopherol decreases the expression of the major scavenger receptor, CD36, in human macrophages via inhibition of tyrosine kinase (Tyk2). *Atherosclerosis,* **2004***; 175(2)*; 213-220.
352. Villacorta L, Graça-Souza AV, Ricciarelli R, Zingg J, Azzi A. Alpha-tocopherol induces expression of connective tissue growth factor and antagonizes tumor necrosis factor-

alpha-mediated downregulation in human smooth muscle cells. *Circ Res,* **2003***; 92(1)*; 104-110.

353. Virtamo J, Rapola JM, Ripatti S, Heinonen OP, Taylor PR, Albanes D, Huttunen JK. Effect of vitamin E and beta carotene on the incidence of primary nonfatal myocardial infarction and fatal coronary heart disease. *Arch Intern Med,* **1998***; 158(6)*; 668-675.

354. Vivekananthan DP, Penn MS, Sapp SK, Hsu A, Topol EJ. Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. *Lancet,* **2003***; 361(9374)*; 2017-2023.

355. Wada S. Cancer preventive effects of vitamin E. *Curr Pharm Biotechnol,* **2012***; 13(1)*; 156-164.

356. Wallert M, Mosig S, Rennert K, Funke H, Ristow M, Pellegrino RM, Cruciani G, Galli F, Lorkowski S, Birringer M. Long-chain metabolites of α-tocopherol occur in human serum and inhibit macrophage foam cell formation in vitro. *Free Radic Biol Med,* **2013 // 2014***; 68*; 43-51.

357. Wallert M, Schmölz L, Galli F, Birringer M, Lorkowski S. Regulatory metabolites of vitamin E and their putative relevance for atherogenesis. *Redox Biol,* **2014**.

358. Wang Y, Miao X, Liu Y, Li F, Liu Q, Sun J, Cai L. Dysregulation of Histone Acetyltransferases and Deacetylases in Cardiovascular Diseases. *Oxid Med Cell Longev,* **2014***;* 641979.

359. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Hörkkö S, Barnard J, Reynolds WF, Topol EJ, DiDonato JA, Hazen SL. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med,* **2007***; 13(10)*; 1176-1184.

360. Watkins ML, Erickson JD, Thun MJ, Mulinare J, Heath CW. Multivitamin use and mortality in a large prospective study. *Am J Epidemiol,* **2000***; 152(2)*; 149-162.

361. Wechter WJ, Kantoci D, Murray ED, D'Amico DC, Jung ME, Wang WH. A new endogenous natriuretic factor: LLU-alpha. *Proc Natl Acad Sci U.S.A.,* **1996***; 93(12)*; 6002-6007.

362. Weiser H, Riss G, Kormann AW. Selective biodiscrimination of alpha-tocopherol stereoisomers. Similar enrichment of all 2R forms in rat tissues after oral all-rac-alpha-tocopheryl acetate. *Ann N Y Acad Sci,* **1992***; 669*; 393-395.

363. Weiser H, Riss G, Kormann AW. Biodiscrimination of the eight alpha-tocopherol stereoisomers results in preferential accumulation of the four 2R forms in tissues and plasma of rats. *J Nutr,* **1996***; 126(10)*; 2539-2549.

364. Whittle KJ, Dunphy PJ, Pennock JF. Plastochromanol in the leaves of Hevea brasiliensis. **1965**.

365. Williams RJ, Motteram JM, Sharp CH, Gallagher PJ. Dietary vitamin E and the attenuation of early lesion development in modified Watanabe rabbits. *Atherosclerosis,* **1992***; 94(2-3)*; 153-159.

366. Wong RSY, Radhakrishnan AK. Tocotrienol research: past into present. *Nutrition Reviews,* **2012***; 70(9)*; 483-490.

367. Wright ME, Lawson KA, Weinstein SJ, Pietinen P, Taylor PR, Virtamo J, Albanes D. Higher baseline serum concentrations of vitamin E are associated with lower total and

cause-specific mortality in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. *Am J Clin Nutr,* **2006***; 84(5)*; 1200-1207.

368. Wu D, Koga T, Martin KR, Meydani M. Effect of vitamin E on human aortic endothelial cell production of chemokines and adhesion to monocytes. *Atherosclerosis,* **1999***; 147(2)*; 297-307.

369. Wu D, Liu L, Meydani M, Meydani SN. Vitamin E increases production of vasodilator prostanoids in human aortic endothelial cells through opposing effects on cyclooxygenase-2 and phospholipase A2. *J Nutr,* **2005***; 135(8)*; 1847-1853.

370. Wu D, Pan W. GSK3: a multifaceted kinase in Wnt signaling. *Trends Biochem Sci,* **2010***; 35(3)*; 161-168.

371. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, De Nardo D, Gohel TD, Emde M, Schmidleithner L, Ganesan H, Nino-Castro A, Mallmann MR, Labzin L, Theis H, Kraut M, Beyer M, Latz E, Freeman TC, Ulas T, Schultze JL. Transcriptome-Based Network Analysis Reveals a Spectrum Model of Human Macrophage Activation. *Immunity,* **2014***; 40(2)*; 274–288.

372. Yam M, Abdul H, Sitti R, Cheng H, Nesaretnam K. Tocotrienols Suppress Proinflammatory Markers and Cyclooxygenase-2 Expression in RAW264.7 Macrophages. *Lipids,* **2009***; 44(9)*; 787-797.

373. Yamamoto Y, Fujisawa A, Hara A, Dunlap WC. An unusual vitamin E constituent (-tocomonoenol) provides enhanced antioxidant protection in marine organisms adapted to cold-water environments. *Proceedings of the National Academy of Sciences,* **2001***; 98(23)*; 13144-13148.

374. Yap SP, Yuen KH, Wong JW. Pharmacokinetics and bioavailability of alpha-, gamma- and delta-tocotrienols under different food status. *J Pharm Pharmacol,* **2001***; 53(1)*; 67-71.

375. Yasunari K, Kohno M, Kano H, Yokokawa K, Minami M, Yoshikawa J. Antioxidants improve impaired insulin-mediated glucose uptake and prevent migration and proliferation of cultured rabbit coronary smooth muscle cells induced by high glucose. *Circulation,* **1999***; 99(10)*; 1370-1378.

376. You C, Sontag TJ, Swanson JE, Parker RS. Long-chain carboxychromanols are the major metabolites of tocopherols and tocotrienols in A549 lung epithelial cells but not HepG2 cells. *J Nutr,* **2005***; 135(2)*; 227-232.

377. Yu SG, Thomas AM, Gapor A, Tan B, Qureshi N, Qureshi AA. Dose-response impact of various tocotrienols on serum lipid parameters in 5-week-old female chickens. *Lipids,* **2006***; 41(5)*; 453-461.

378. Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med,* **2000***; 342(3)*; 154-160.

379. Zhao Y, Lee M, Cheung C, Ju J, Chen Y, Liu B, Hu L, Yang CS. Analysis of Multiple Metabolites of Tocopherols and Tocotrienols in Mice and Humans. *J Agric Food Chem,* **2010***; 58(8)*; 4844-4852.

380. Zingg J. Modulation of signal transduction by vitamin E. *Mol Aspects Med,* **2007***; 28(5-6)*; 481-506.

381. Zingg J, Libinaki R, Lai C, Meydani M, Gianello R, Ogru E, Azzi A. Modulation of gene expression by α-tocopherol and α-tocopheryl phosphate in THP-1 monocytes. *Free Radic Biol Med,* **2010a***; 49(12)*; 1989-2000.

382. Zingg J, Libinaki R, Meydani M, Azzi A. Modulation of phosphorylation of tocopherol and phosphatidylinositol by hTAP1/SEC14L2-mediated lipid exchange. *PLoS ONE,* **2014**.

383. Zingg J, Meydani M, Azzi A. α-Tocopheryl phosphate - An active lipid mediator? *Mol Nutr Food Res,* **2010b***; 54(5)*; 679-692.

B Supplement

Supplement to:

Long-chain metabolites of α-tocopherol occur in human serum and inhibit macrophage foam cell formation *in vitro*

Supplementary Material

Supplementary Table T1

Human CD36 and RPL37A primer sequences used for quantitative real-time-PCR analyses.

primer	forward	reverse
hCD36	5'-TCA CTG CGA CAT GAT TAA TGG TAC A-3'	5'-ACG TCG GAT TCA AAT ACA GCA TAG AT-3'
hRPL37A	5'-ATT GAA ATC AGC CAG CAC GC-3'	5'-AGG AAC CAC AGT GCC AGA TCC-3'

Supplementary Material

Supplementary Figure S1

Hepatic metabolism of α-tocopherol. Long-chain metabolites of α-TOH (α-13'-OH, α-13'-COOH) are formed by side-chain truncation and conjugation. Tocopherols are metabolized in the liver *via* an initial CYP3A4-dependent ϖ-hydroxylation, which results in the formation of the alcohol derivative α-13'-OH. Subsequent β-oxidation in peroxisomes forms the acid derivative α-13'-COOH. The major short-chain metabolite of α-TOH and end-product of α-TOH catabolism is α-CEHC. **(1)** α-TOH (2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-6-chromanol), **(2)** α-13'-OH (13'-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanol), **(3)** α-13'-COOH (13'-(6-hydroxy-2,5,7,8,-tetramethylchroman-2-yl)-2,6,10-trimethyl-tridecanoic acid), and **(4)** α-CEHC (2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman).

Supplementary Figure S2

Time-dependency of oxLDL uptake by THP-1 macrophages. A time-course experiment was performed to determine optimal conditions for measuring the uptake of oxLDL at 37°C. THP-1 macrophages were incubated with 50 mg/l oxidized LDL (oxLDL) for 0 h, 15 min, 30 min, 1 h, 2 h, 4 h or 6 h using a mixture of 10 mg/l AlexaFluor488-labeled oxLDL and 40 mg/l of unlabeled oxLDL.

Supplementary Figure S3

Induction of CD36 mRNA by α-13'-OH and α-13'-COOH is dose-dependent. THP-1 macrophages were incubated with 0 µmol/l, 1 µmol/l, 2 µmol/l, 5 µmol/l, 10 µmol/l, 20 µmol/l α-13'-OH or 0 µmol/l, 0.5 µmol/l, 1 µmol/l, 2 µmol/l, 5 µmol/l, 7.5 µmol/l α-13'-COOH for 24 h in serum-free culture medium. Changes in CD36 mRNA were quantified using RT-qPCR and results were normalized to the reference gene RPL37A. Expression of the reference genes was not affected at the concentrations used. Columns represent mean of relative fold changes and bars display calculated maximum and minimum expression levels representing

SEM expression levels of three independent biological samples performed in two technical replicates. **, $p < 0.01$; ***, $p < 0.001$ (*vs.* no treatment)

Supplementary Figure S4

α-13'-OH and α-13'-COOH inhibit uptake of oxLDL in THP-1 macrophages time-dependently. Uptake of AlexaFluor488-labeled oxLDL was measured as increase of fluorescence intensity using flow cytometry at different times as indicated. A mixture of 10 mg/l AlexaFluor488-labeled oxLDL and 40 mg/l unlabeled oxLDL was applied. Mature THP-1 macrophages were pre-incubated for 24 h with α-13'-OH (10 µmol/l) or α-13'-COOH (5 µmol/l). 50 mg/l of the oxLDL mixture were then added to the culture medium for 0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h or 24 h. Bars indicate means ± SD of two independent biological replicates.

Supplementary Figure S5

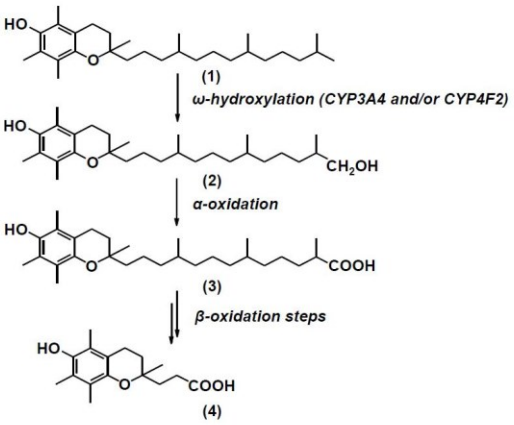
Decrease in uptake of oxLDL by α-TOH LCMs α-13'-OH and α-13'-COOH is independent from CD36. Fluorescence intensity represents the amount of internalized AlexaFluor488-labeled oxLDL. OxLDL uptake was significantly decreased by α-13'-OH and α-13'-COOH by 9 % ± 9 % and 14 % ± 16 %, respectively, compared to oxLDL uptake in the absence of any compound. OxLDL uptake by THP-1 macrophages was partly and significantly inhibited by CD36 blocking antibody by 23 % ± 11 %. Pre-incubation with CD36 blocking antibody did not affect the LCM-mediated decrease in oxLDL uptake by (α-13'-OH, 16 % ± 11 %; α-13'-COOH, 14 % ± 14 %). Bars indicate means ± SD of nine independent experiments. *, $p < 0.05$; ***, $p < 0.001$ (*vs.* no treatment)

Supplementary Figure S6

α-13'-OH and α-13'-COOH inhibit phagocytosis in macrophages time-dependently. Phagocytic uptake of fluorescence-labeled microbeads was measured by flow cytometry. THP-1 macrophages were pre-incubated for 24 h with 10 µmol/l α-13'-OH or 5 µmol/l α-13'-COOH followed by addition of fluorescence-labeled microbeads to the culture medium for

1 h, 2 h, 4 h, 6 h, 8 h, 12 h or 24 h. Non-treated cells served as controls. Bars indicate means ± SD of two independent biological replicates.

Supplement Figure 1



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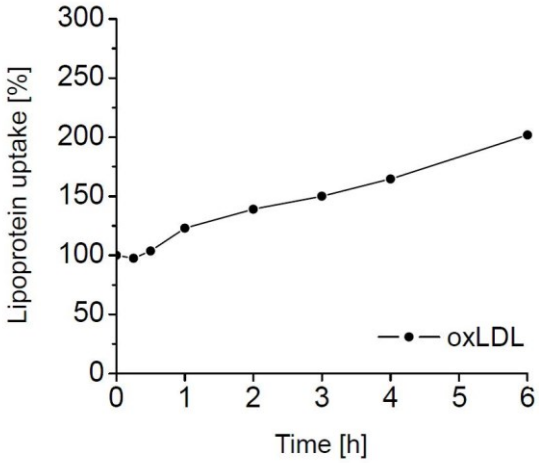

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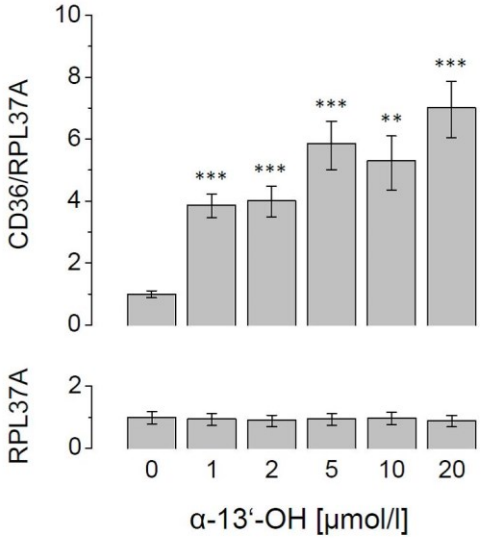

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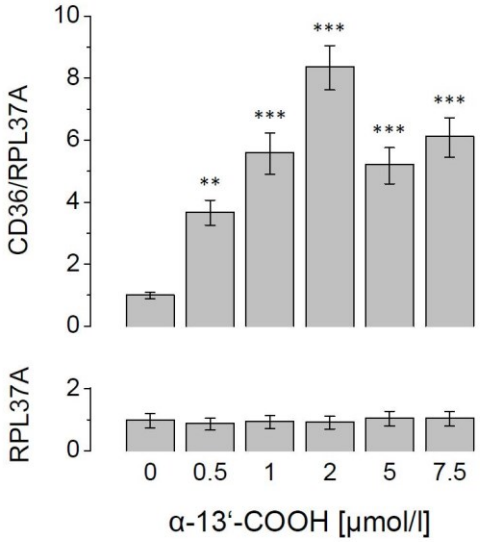

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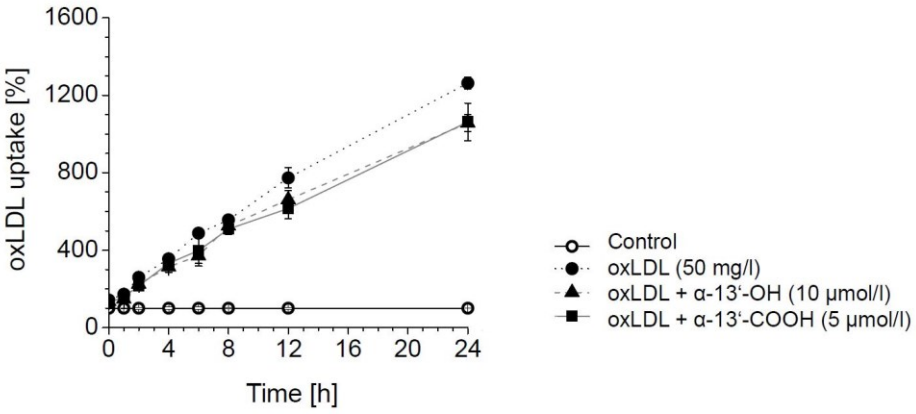

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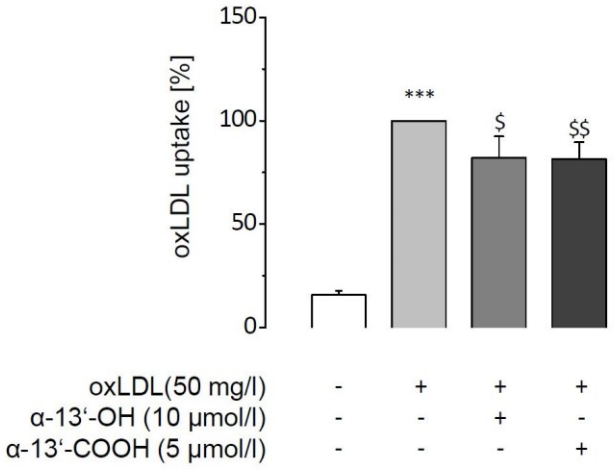

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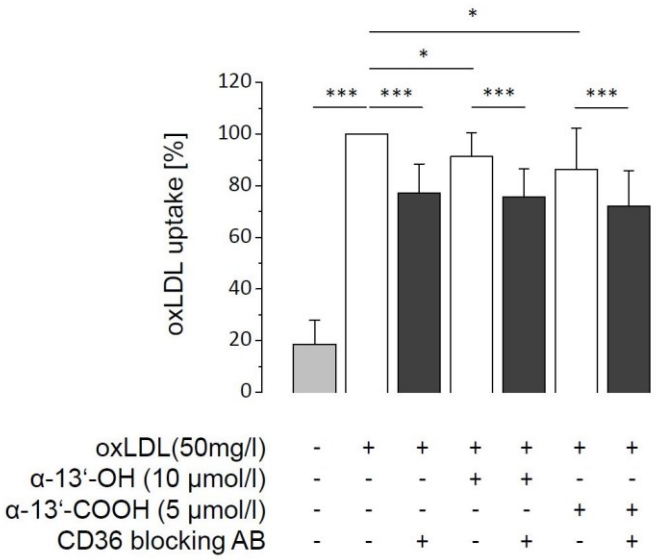

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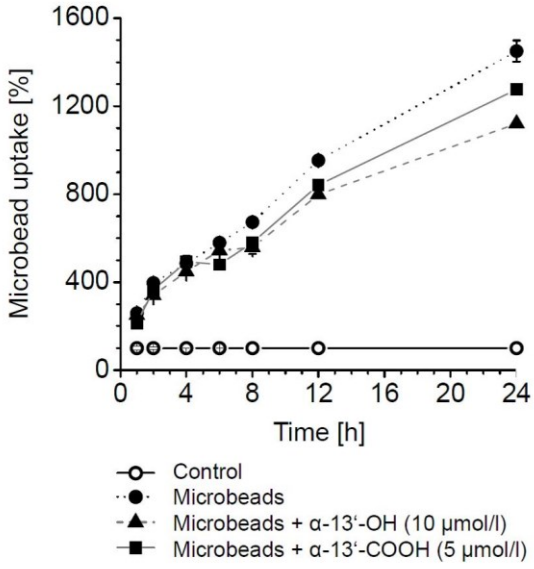

Supplement to:

The α-tocopherol long-chain metabolite α-13'-COOH affects the inflammatory burst of lipopolysaccharide-activated murine RAW264.7 macrophages

Table 1: PCR primers used in this study. In each case, forward and reverse primers are located in different exons.

mRNA	mRNA name	GenBank accession no.	Forward primer	Reverse primer	Amplicon size [bp]
Cox2/ mPtgs2	Cyclooxygenase 2	NM_011198	TCCCTGAAGCCGTACACATCA	TGGACGAGGTTTTTCCACCA	132
Il1β	Interleukin 1beta	NM_008361.3	TGAAGTTGACGGACCCCAAA	CAGCCACAATGAGTGATACTGCC	140
Il6	Interleukin 6	NM_031168.1	TCAATTCCAGAAACCGCTATGAA	GGAAGGCCGTGGTTGTCAC	94
Il10/Csif	Interleukin 10	NM_010548.2	AAATAAGAGCAAGGCAGTGGAGC	TCATTCATGGCCTTGTAGACACC	81
iNos/ Nos2	Inducible Nitric oxide synthases	NM_010927.3	GAGCGAGGAGCAGGTGGAA	CCATAGGAAAAGACTGCACCGA	90
Ppib	Peptidylprolyl Isomerase B	NM_011149.2	AAACAGCAAGTTCCATCGTGTCAT	GAAGCGCTCACCATAGATGCTCT	103
Tnfα	Tumor necrosis factor α	NM_013693	AGAAACACAAGATGCTGGGACAGT	CCTTTGCAGAACTCAGGAATGG	46

B Danksagung

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C Curriculum vitae

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Name	**Maria Wallert**
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Education

Since January 2010	**PhD thesis** **Regulation of non-antioxidative and inflammatory pathways in macrophages by long-chain metabolites of α-tocopherol** (Supervisor: Prof. Dr. Stefan Lorkowski), Department Biochemistry of Nutrition, Institute of Nutrition, Friedrich Schiller University Jena
November 2009	**Diploma in Nutrition Sciences** (German equivalent of Master of Science: Diplom-Trophologin), Grade: 1.3
May - November 2009	**Diploma thesis** **Biological effects of vitamin E metabolites on human monocytes and macrophages** (German: Die biologische Wirkung von Vitamin E-Metaboliten auf humane Monozyten und Makrophagen) (Supervisor: Prof. Dr. Stefan Lorkowski), Department Biochemistry of Nutrition, Institute of Nutrition, Friedrich Schiller University Jena
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October 2004 – November 2009	**Studies of Nutritional Science** Friedrich Schiller University Jena
June 2004	**German University Entrance Qualification** (German equivalent: Allgemeine Hochschulreife), Grade: 1.7

External Research Placements

June-August 2012	**Workgroup Prof. Dr. Francesco Galli** Nutrition and clinical biochemistry laboratory, Department of Internal Medicine, School of Pharmacy, University of Perugia, Italy
December 2012	**Workgroup Prof. Dr. Marc Birringer** University of Applied Sciences, Department of Nutritional, Food and Consumer Studies, Fulda

Awards

April 2014	**Travel grant** Participation at 3rd International Vitamin Conference (IVC) Waters Corporation and Agilent Technologies, MA, USA
March 2014	**Prochance – Program line A2** Participation at 3rd International Vitamin Conference (IVC)
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March 2012	**Travel Award** Alumni & Partner of the Friedrich Schiller University, Science in Nutrition/Lifesciences (**APFEL**) e.V. Participation at the Annual Congress of the German Society of Nutrition (DGE)
March 2012	**Fellowship** of the German Academic Exchange Service (Deutscher Akademischer Austauschdienst, **DAAD**) Participation at the 15th International Meeting on Fat Soluble Vitamins (FSV)
March 2012	**Young Investigator Award** for the best scientific congress contribution at the 15th International Meeting on Fat Soluble Vitamins (FSV), Kalabaka, Greece
March 2011	**German Alpro Foundation Award** for the best German Diploma thesis: Biological effects of vitamin E metabolites on human monocytes and macrophages
November 2010	**Award** of the Alumni & Partner of the Friedrich Schiller University, Science in Nutrition/Lifesciences (**APFEL**) e.V. for outstanding Diploma thesis: Biological effects of vitamin E metabolites on human monocytes and macrophages

Professional Memberships

Since 2010	Alumni & Partner of the Friedrich Schiller University, Science in Nutrition/Lifesciences (**APFEL**) e. V.
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D Publications

Original Publications and Reviews

Wallert M, Schmölz L, Galli F, Birringer M, Lorkowski S. Regulatory metabolites of vitamin E and their putative relevance for atherogenesis. *Redox Biol* 2014; 2:495-503.

Wallert M, Mosig M, Rennert K, Funke H, Ristow M, Galli F, Lorkowski S, Birringer M. Long-chain metabolites of α-tocopherol occur in human serum and inhibit macrophage foam cell formation in vitro. *Free Radic Biol Med* 2014; 68C:43-51.

Scientific Abstracts

Wallert M, Schmölz L, Heise J, Krauth V, Werz O, Birringer M, Lorkowski S. Long-chain metabolites of α-tocopherol and a δ-tocotrienol-related natural compound regulate inflammatory pathways in macrophages. *Perfusion* 2014; 27(1):17-18.

Heise J, **Wallert M**, Schmölz, L, Krauth V, Werz O, Birringer M, Lorkowski S. Garcinoic acid, a δ-tocotrienol-related natural product, reduces inflammatory response of macrophages. *Perfusion* 2014; 27(1):34.

Schmölz L, **Wallert M**, Birringer M, Lorkowski S. Long-chain metabolites of vitamin E protects human macrophages against lipotoxicity. *Perfusion*, 2014; 27(1):34-35.

Kluge S, Schmölz L, **Wallert M**, Birringer M, Lorkowski S. α-Tocopherol long-chain metabolites regulate the expression of ABCA1 in primary fibroblasts. *Perfusion* 2014; 27(1):35.

Wallert M, Schmölz L, Becher S, Birringer M, Lorkowski S. Regulation of adipophilin by long-chain metabolites of α-tocopherol in macrophages is mediated via MAPK pathways. *Perfusion* 2013; 26(2):68.

Schmölz L, **Wallert M**, Birringer M, Lorkowski S. Investigations on the relationship of structure to effectiveness of tocopherols and their long-chain metabolites. *Perfusion*, 2013; 26(2):68.

Wallert M, Schmölz L, Lütjohann D, Mosig S, Rennert K, Birringer M, Lorkowski S. Antioxidative and non-antioxidative effects of tocopherol metabolites on atherogenesis. Annals of Nutrition & Metabolism 2012; 60:131-145.

Wallert M, Schmölz L, Lütjohann D, Mosig S, Rennert K, Birringer M, Lorkowski S. Regulation of cholesterol homeostasis by α-TOH and its long-chain metabolites. Conference Report of the German Society of Nutrition 2012; 17:119.

Becher S, **Wallert M**, Maeß MB, Rennert K, Mosig S, Grün M, Lorkowski S. PPARγ antagonist GW9662 triggers PLIN2 expression and triglyceride accumulation in human THP-1 macrophages. Conference Report of the German Society of Nutrition 2012; 17:60.

Wallert M, Sarem Z, Becher S, Birringer M, Lorkowski S. Tocopherols – Side-chain structure is important for non-antioxidative effects. *Perfusion* 2012; 25(1):34.

Becher S, **Wallert M**, Maeß MB, Rennert K, Mosig S, Grün M, Lorkowski S. A fraction of the action: new insights in how GW9662 affects lipid metabolism in THP-1 macrophages. *Perfusion* 2012; 25(1):31.

Maeß MB, **Wallert M**, Becher S, Lorkowski S. PMA-induced activation of THP-1 macrophages using standard differentiation protocols. *Perfusion* 2012; 25(1):30-31.

Schmölz L, **Wallert M**, Lütjohann D, Mosig S, Rennert K, Birringer M, Lorkowski S. α-Tocopherol regulates cholesterol homeostasis - What about long chain metabolites? *Perfusion* 2012; 25(1):34-35.

Wallert M, Mosig S, Rennert K, Birringer M, Lorkowski S. Long-chain metabolites of α-TOH regulates inflammatory pathways. *Perfusion* 2011; 24(1):30.

Sendelbach S, **Wallert M**, Maeß MB, Grün M, Lorkowski S. PPARγ antagonist GW9662 modulates macrophage function. *Perfusion* 2011; 24(1):29.

Wallert M, Sarem Z, Sendelbach S, Birringer M, Lorkowski S. Effects of δ-tocopherol long-chain metabolites on macrophage foam cell formation. *Perfusion* 2011; 24(1):38.

Wallert M, Mosig S, Rennert K, Funke H, Birringer M, Lorkowski S. Long-chain metabolites of α-tocopherol modulates macrophage foam cell formation. *Perfusion* 2011; 24(1):37.

Wallert M, Lorkowski S, Mosig S, Rennert K, Funke H, Ristow M, Birringer M. Antiinflammatorische Tocopherolmetabolite hemmen die LPS-induzierte NO-Produktion in Mausmakrophagen. Conference Report of the German Society of Nutrition 2011; 15:84.

Wallert M, Mosig S, Rennert K, Funke H, Keller S, Jahreis G, Birringer M, Lorkowski S. Tocopherol metabolites and atherogenesis – new light on an old story? *Perfusion* 2010; 23(1):33-34.

Wallert M, Mosig S, Rennert K, Lorkowski S, Birringer M. Biological activity of tocopherol metabolites with a special focus on iNOS. *Conference Report of the Signal Transduction Society* 2010:153.

Wallert M, Lorkowski S, Mosig S, Rennert K, Funke H, Ristow M, Birringer M. Biological activity of tocopherol metabolites. *Conference Report of the Signal Transduction Society* 2009:155.

E Eidesstattliche Erklärungen

Hiermit erkläre ich an Eidesstatt, dass:

(i) mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist.

(ii) ich die vorliegende Dissertation selbst angefertigt habe und keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen habe und alle benutzen Hilfsmittel, persönliche Mitteilungen und Quellen in der Arbeit angegeben habe.

(iii) mich lediglich die in den Manuskripten angegebenen Personen bei der Auswahl und der Auswertung des Materials sowie bei der Erstellung der Manuskripte unterstützt haben.

(iv) ich nicht die Hilfe eines Promotionsberaters in Anspruch genommen habe.

(v) Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

(vi) ich diese Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe.

(vii) ich weder die gleiche oder eine in wesentlichen Teilen ähnliche, noch eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, Juni 2014 _____

Maria Wallert